Univerzita Karlova

Přírodovědecká fakulta

Studijní program:

Vývojová a buněčná biologie

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Wnt/β-kateninová signalizace ve vývoji mořského kroužkovce *Platynereis dumerilii*

Wnt/β-catenin signalling in the development of the marine annelid *Platynereis dumerilii*

Disertační práce

Vedoucí práce/Školitel:

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Praha 2018

Charles University

Faculty of Science

Závazné prohlášení:

Prohlašuji, že předloženou disertační práci jsem vypracoval samostatně. Výsledků bylo dosaženo prací ve vědeckém týmu RNDr. Zbyňka Kozmika, CSc. na Ústavu molekulární genetiky, v. v. i. Akademie věd České republiky. RNDr. Zbyněk Kozmik, CSc. navrhl projekt a experimenty, konzultoval výsledky a pomohl sepsat přiloženou publikaci. RNDr. Ondřej Machoň, CSc. navrhl a provedl izolaci prvních fragmentů *Pdu-Tcf*. Chrysoula Pantzartzi, Ph.D. pomohla lokalizovat C-clamp (+) variantu *Pdu-Tcf* a podílela se na analýze jeho sekvence. Mgr. Radim Žídek se podílel na návrhu experimentů, provedl experimenty, získal a zpracoval data a sepsal přiloženou publikaci. Všechny použité zdroje a literatura byly řádně citovány. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze,

Mgr. Radim Žídek

I want to dedicate this work to my parents and grand-parents, who have all been encouraging my passion for nature from my early childhood. They were an indispensable support throughout my studies. A special dedication comes to my grandmother Vojtěška, who passed away before this work was completed. You will never be forgotten.

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aa	amino acid
AcTub	acetylated tubulin
AP	alkaline phosphatase
A-P	anterior-posterior (axis)
APC	adenomatous polyposis coli (protein)
A-V	animal-vegetal (axis)
BCIP	5-bromo-4-chloro-3‘-indolylphosphate
bHLH	basic helix-loop-helix (transcription factor)
BMP(s)	bone morphogenetic protein(s)
BSA	bovine serum albumin
Cdx	a homologue to *Caudal* homeobox
CK1α/γ	Casein kinase 1 α or γ
CNS	central nervous system
CRD	cysteine-rich domain (of Frizzled of sFRP)
CtBP1	C-terminal binding protein 1
DAPI	4′,6-diamidino-2-phenylindole
DEPC	diethyl-pyrocarbonate
DIG	digoxigenin
DMSO	dimethylsulphoxide
dpf	days post-fertilization
D-V	dorsal-ventral (axis)
EdU	5-ethynyl-2’-deoxyuridine
En	Engrailed
ER	endoplasmic reticulum
EtOH	ethanol
FGF	fibroblast growth factor
FNSW	filtered natural sea water
Fz	Frizzled (receptor of Wnt)
GBS	Groucho binding sequence
GRN	gene regulatory network
GSK-3β	Glycogen synthase kinase 3 beta
Hh	Hedgehog (protein)

HIPK2	homeodomain-interacting protein kinase 2
HMG-box	high mobility group box (protein DNA-binding domain)
HMG DBD	HMG-box DNA-binding domain (of Tcf, HMG-box + basic tail)
HNF	hepatocyte nuclear factor
hpf	hours post-fertilization
HPSGs	heparan sulphate proteoglycans
HybMix	hybridization mixture/buffer (for in situ RNA hybridization)
ICAT	inhibitor of β-catenin and TCF-4
ISCs	intestinal stem cells
IsO	isthmic organizer
LCA	last common ancestor
LRP5/6	LDL (low density lipoprotein)-receptor-related protein 5 or 6/Arrow
MABT	maleic acid buffer + Tween20
MetOH	methanol
MHB	midbrain-hindbrain boundary
mpf	months post-fertilization
MAB	maleic acid buffer
MIP	myoinhibitory peptide
NBT	4-nitro blue tetrazolium
Ngn	Neurogenin
NSW	natural sea water
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdu	*Platynereis dumerilii* (in gene names)
PFA	paraformaldehyde
PGZ	posterior growth zone
PIP2	phosphatidylinositol-4,5-bisphosphate
Ptch	Patched (the receptor of Hedgehog)
PTw	phosphate buffered saline + Tween20
SAZ	segment addition zone
SCF	Skp, Cullin, F-box-containing E3 ubiquitin ligase complex
SDS	sodium dodecyl sulphate
SDS-PAAGE	sodium dodecyl sulphate polyacryl amide gel electrophoresis

Shh	Sonic hedgehog
SSCT	saline-sodium citrate + Tween20
TAE	Tris-acetate-EDTA buffer
Tcf	T-cell factor
TDE	2,2'-Thiodiethanol
TF	transcription factor
TGF-β	transforming growth factor- β
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VNCs	ventral nerve cords
Wg	Wingless (protein)
Wnt	Wingless-type MMTV integration site (original abbreviation)
ZLI	zona limitans intrathalamica

5 Preface

This thesis contains most of the data that I have collected from three of my projects on the same experimental model, the marine annelid worm *Platynereis dumerilii*. They all concerned with the role of Wnt/β-catenin signalling pathway in three different organ systems or developmental processes – the neuroectoderm of the central nervous system, the segmentation of the body and the gut development. The data from the former two were still incomplete and unpublished by the time of writing of this thesis. But I decided to include them here anyway, as they already allowed me to formulate hypotheses that answer the research questions. The inclusion of all three projects puts the function of Wnt/β-catenin signalling in *Platynereis* into a broader developmental context. As I will later, this approach enables to disentangle problems that would otherwise remain enigmatic if studied isolatedly.

Accordingly, the introduction to the thesis had to be very broad and detailed to cover all three topics and trace them to their initial developmental causes. Nevertheless, all events in the embryonic development are interconnected and the more information we have, the more connections we can see.

I also include here several experiments and results that represented blind ends or at first looked like they did. However, they also confer some informational value which was often revealed only after the examination and interpretation of the complete data.

The evolutionary developmental biology uses a comparative approach to development of various organisms to make evolutionary inferences. As such, it faces a problem of the non-uniformity of nomenclature from these various sources. In this thesis, I was often forced to refer to genes and proteins from several different organisms. Because there is an inconsistency in the usage of capitals and italics and there are historical exceptions from these rules, I tried to unify the nomenclature to the one I used in the article that is attached to this thesis. Therefore, the italics are used to refer to a gene and the regular font refers to a protein. First capital letters are used in both, although I am aware that it is more frequent to use it only for proteins. In some cases, I use two synonyms for one gene/protein, if both names are widely used (typically insects vs. chordates) but I always state this equivalency on the first use. The genes and proteins of *Platynereis dumerilii* are introduced by an abbreviation *Pdu*-/Pdu- if not referred to more organisms at once.

6 Abstract

6.1. Abstract (in English)

Wnt/β-catenin signalling is absolutely crucial for the early embryonic development of metazoan animals from the establishment of body axes, through the specification of germ layers and tissues to the development of organ systems. I used pharmacological manipulations of the Wnt/β-catenin pathway activity in the planktonic larvae of the marine polychaete annelid *Platynereis dumerilii*, the representative of the clade Spiralia, to investigate the role of Wnt/β-catenin signalling in the development and evolution of three hallmarks of Bilateria: the central nervous system, the body segmentation and the digestive tube.

Wnt proteins are produced in all three aforementioned systems in *Platynereis* where they trigger the Wnt/β-catenin pathway in neighbouring cells. I describe here, for the first time in *Platynereis*, a homologue of the endpoint transcription factor of the entire pathway, *Pdu-Tcf*, which is subjected to an alternative splicing and along with a Wnt target gene *Pdu-Axin* is expressed in tissues with the active Wnt signalling – in the brain ganglia, in the neuroectoderm along the ventral midline, in segments, in the posterior growth zone and in the gut.

Pharmacological manipulations suggest that Wnt/β-catenin signalling specifies neuronal progenitors in the ectoderm and promotes their proliferation, but it is not involved in the patterning of the nervous system in *Platynereis* as it does not significantly shift the boundaries of the expression domains of the neural-specific transcription factors. However, an analysis of their normal expression revealed a putative homology of the vertebrate and insect brain signalling centre, the isthmic organizer, with the ciliated posterior boundary of peristomium with the cryptic zero segment and suggested the existence of another in the anterior peristomium boundary. I thus propose that organizers of brain development are derived from the ciliated bands of an ancient planktonic bilaterian ancestor.

Wnt/β-catenin signalling positively regulates the segmentation gene *Pdu-Engrailed* on the intersegmental boundary, which confirms the current model of segmentation in *Platynereis* by the mechanism that is conserved between *Platynereis* and *Drosophila* and suggests a presence of a mutually exclusive, non-autonomous positive feedback loop between Wnt and Hedgehog signalling pathways. The over-activation of the Wnt/β-catenin pathway leads to a loss of chaetal sacs and of morphological, but not molecular boundaries

between segments. The Wnt/β-catenin signalling is active also in the posterior growth zone where it is probably involved in the formation of new segments.

The midgut development is delayed relative the anterior and posterior parts of the gut due to a high amount of yolk in the macromeres that inhibits cell division. It is reactivated much later in the nectochaete stage and entails the expression of neural-specific transcription factors *Pdu-Otx* and *Pdu-Nk2.1*. Wnt/β-catenin signalling positively regulates *Pdu-Cdx* in the hindgut and previously unrecognized domains in the ventral gut midline and the midgut/foregut boundary. The inhibition of Wnt/β-catenin pathway completely blocks the proliferation in the entire body of the larva and arrests the differentiation of the gut endoderm to a digestive epithelium. The typical expression of digestive enzymes diminishes from the midgut which instead retains the expression of *Pdu-Legumain* which is expressed here earlier, but is normally in this phase present already only in the hindgut. This state is not permanent and the differentiation continues once the inhibition is alleviated. I propose that the Wnt/β-catenin signalling specifies endodermal gut progenitors, promotes their proliferation and triggers steps that eventually lead to their differentiation.

Keywords:
Wnt signalling, development, evolution, neuroectoderm, brain, segmentation, gut, Tcf, Hox, Pax, Protostomia, Annelida

6.2. Abstrakt (česky)

Wnt/β-kateninová signalizace je zásadní pro raný embryonální vývoj mnohobuněčných živočichů, od ustavení tělních os, přes určení zárodečných listů a tkání až po vývoj orgánových soustav. Použil jsem farmakologické ovlivnění aktivity Wnt/β-kateninové dráhy u planktonních larev mořského mnohoštětinatého kroužkovce, nereidky *Platynereis dumerilii*, zástupce skupiny Spiralia, abych prošetřil úlohu Wnt/β-kateninové signalizace ve vývoji a evoluci tří charakteristických znaků dvoustraně souměrných živočichů (Bilateria): centrální nervové soustavy, tělního článkování a trávicí trubice.

Wnt proteiny jsou u nereidky produkovány ve všech tří výše zmíněných soustavách, kde spouští Wnt/β-kateninovou dráhu v sousedních buňkách. Vůbec poprvé zde u nereidky popisuji homolog koncového transkripčního faktoru celé dráhy, *Pdu-Tcf*, který je předmětem alternativního sestřihu a spolu s cílovým genem Wnt signalizace, *Pdu-Axin*em, exprimován v tkáních s aktivní Wnt signalizací – v mozkových gangliích, v neuroektodermu podél břišní středové linie, v článcích, v posteriorní růstové zóně a ve střevě.

Farmakologické manipulace naznačují, že Wnt/β-kateninová signalizace v ektodermu nereidky specifikuje progenitory neuronů a podporuje jejich proliferaci, ale není zapojena do členění nervového systému, jelikož výrazně neposouvá hranice expresních domén neurospecifických transkripčních faktorů. Analýza jejich normální exprese odhalila pravděpodobnou homologii signálního centra obratlovčího a hmyzího mozku, isthmického organizátoru, s obrvenou zadní hranicí peristomia a kryptického nultého segmentu a napověděla existenci homologu dalšího organizátoru na přední hranici peristomia. Předkládám hypotézu, podle níž jsou organizátory vývoje mozku odvozeny od obrvených pásů dávného planktonního předka dvoustraně souměrných živočichů.

Wnt/β-kateninová signalizace pozitivně reguluje segmentační gen *Pdu-Engrailed* na hranici mezi segmenty, což potvrzuje současný model segmentace u nereidky mechanismem, který je zachován mezi nereidkou a octomilkou a značí přítomnost vzájemně výlučné neautonomní pozitivní zpětnovazebné smyčky mezi Wnt a Hedgehog signalizací. Nadměrná aktivace Wnt/β-kateninové dráhy vede ke ztrátě vaků se štětinami a morfologických, avšak nikoliv molekulárních hranic mezi segmenty. Wnt/β-kateninová signalizace je taktéž aktivní v posteriorní růstové zóně, kde je pravděpodobně zapojena do tvorby nových článků.

Vývoj středního střeva je opožděn v porovnání s jeho přední a zádní částí kvůli velkému množství žloutku v makromerách, které brání buněčnému dělení. Jeho vývoj je znovu aktivován mnohem později ve stádiu nektochéty a zahrnuje expresi neurálních transkripčních faktorů *Pdu-Otx* a *Pdu-Nk2.1*. Wnt/β-kateninová signalizace pozitivně reguluje *Pdu-Cdx* v zadním střevě a v dříve nerozpoznaných doménách ve ventrální středové linii středního střeva a na hranici předního a zadního střeva. Inhibice Wnt/β-kateninové dráhy v celém těle larvy zcela zablokuje proliferaci a zastaví diferenciaci střevního endodermu v trávicí epitel. Typická exprese trávicích enzymů ze středního střeva vymizí, a to si namísto nich podrží expresi *Pdu-Legumain*u, který je zde exprimován dříve, ale v této fázi je normálně přítomen již pouze v zadním střevu. Tento stav však není stálý a diferenciace pokračuje, jakmile je inhibice uvolněna. Navrhuji, že Wnt/β-kateninová signalizace specifikuje endodermální střevní progenitory, podněcuje jejich proliferaci a spouští kroky, které posléze vedou k jejich diferenciaci.

Klíčová slova:
Wnt signalizace, vývoj, evoluce, neuroektoderm, mozek, segmentace, střevo, Tcf, Hox, Pax, prvoústí, kroužkovci

7 Background

7.1. Wnt signalling

Wnt signalling is one of the major systems of cell-to-cell communication used in the metazoan development. There is only a handful of such signalling pathways and Wnt signalling as one of them along with Hedgehog (Hh), BMP/TGF-β, Nodal, Delta-Notch, FGF, Jak-STAT and MAPK signalling pathways is involved in essentially every aspect of animal embryogenesis and the maintenance of adult tissues. During development, it provides positional information and specifies main body axes (chapter 7.2.1), thereby coordinating proliferation, differentiation and cell fate decisions to regulate tissue diversification, organ growth and shape. In the adulthood, it is important for the maintenance of stem cells, tissue renewal and regeneration.

7.1.1. Overview of Wnt signalling pathways

Despite being sometimes referred to simply as the "Wnt signalling", it in fact encompasses a complex of several signalling pathways with a wide range of actions that can be divided to the control of transcription of target genes (resulting in stem cell maintenance, cell cycle progression, specification of cell types etc.) and the regulation of cytoskeleton (and hence cell shape, adhesion and polarity in oriented cell divisions and morphogenetic movements). A uniting feature of all these pathways is an activation by a secreted signalling protein from the Wnt family. Other steps of the individual signalling cascades can vary, but they most often involve a Frizzled receptor of Wnt and a transduction of the signal via cytoplasmic Dishevelled protein.

The first known and perhaps most intensively studied is the so called canonical Wnt/β-catenin pathway, which regulates the expression of target genes via changes in the stability of a transcriptional co-regulator β-catenin. The other group of signalling pathways is referred to as non-canonical and comprises planar cell polarity (PCP) pathway, Rho-dependent pathways, Wnt/protein kinase C (PKC) pathway, Wnt/Ca^{2+} pathway and others (Semënov et al., 2007). The Wnt/β-catenin pathway will be described to a greater detail in following chapters. In the Wnt/PCP pathway, an enrichment of Frizzled receptors on the side of a cell in a direction towards a source of the Wnt signal orients the microtubule cytoskeleton and hence the vesicular trafficking. This polarizes the activity of other non-

canonical Wnt pathways, most of which are dependent on small monomeric GTPases of the Rho family. Combined action of PCP and Rho pathways can lead to the orientation of mitotic spindles and planes of cell divisions, the orientation of basal bodies and the direction of ciliary beating, polarized changes in cell shape, a directed cell movement and cellular outgrowths etc. (Lapébie et al., 2011; Schlessinger et al., 2009; Sokol, 2015). Given the ubiquity of Wnt signalling in developmental processes, the result of its manipulation can be very pleiotropic.

There is a paradigm, that distinct Wnt pathways are associated with certain paralogues of Wnt ligands and their Frizzled receptors (Takada et al., 2005) or they depend on their combination with co-receptors. Nevertheless, there is some cross-reactivity between individual classes of Wnt ligands and the repertoire of Wnt receptors (Agostino et al., 2017). Wnt pathways also partly utilize overlapping sets of signalling proteins (e. g. the Dishevelled protein) in their transduction cascades and can influence each other by feedback loops, which results in a significant crosstalk among these pathways. Wnt signalling thus functions rather as a network than a single pathway (van Amerongen and Nusse, 2009). It is important to keep this on mind while studying a single branch of Wnt signalling, as its disturbance can somewhat affect the whole network. which can be in part responsible for the observed phenotype. This can be largely precluded by manipulating the pathway downstream in its cascade.

7.1.2. Synthesis and secretion of Wnt proteins

Wnts are a class of secreted glycoproteins that can act as signalling molecules over short or long distances to facilitate cell-to-cell communication. There are 13 classes of Wnt genes (Holstein, 2012; Janssen et al., 2010) which can be further divided to canonical (Wg-/Wnt-1- or Wnt-3-like) or non-canonical (Wnt-5-like) according to their preferences to trigger the respective intracellular pathways. Properties and signalling capacity of Wnt proteins are largely dependent on their posttranslational modifications which are established during their synthesis and export from Wnt producing cells.

7.1.2.1. Wnt post-translational modifications

Wnt proteins are synthesized into the lumen of endoplasmic reticulum (ER) where they are N-glycosylated (Kurayoshi et al., 2007; Papkoff et al., 1987) and acylated. Wnts were initially thought to be palmitoylated on the conserved cysteine (Cys77 in murine Wnt3a) and this modification was thought to be necessary for the signalling activity, but not for the secretion of Wnt protein (Komekado et al., 2007; Kurayoshi et al., 2007; Willert et al., 2003). However, these observation were later rivalled by a finding that the corresponding cysteine engages in a disulphide bond in *Xenopus* XWnt8 (Janda et al., 2012). On the other hand, the modification with acyl moiety (reported to be palmitoleic acid, but may be also palmitic) on the serine residue by the O-acyltransferase Porcupine on the serine residue (Ser209 in murine Wnt3a) has been confirmed repeatedly (Janda et al., 2012; Rios-Esteves et al., 2014; Takada et al., 2006). The acylation(s) explain why Wnt proteins are more hydrophobic than expected just from their amino acid sequences and why they mostly remain associated with the cell surface or the extracellular matrix. Either glycosylation (Komekado et al., 2007; Kurayoshi et al., 2007) and serine acylation (Takada et al., 2006) are required for Wnt proteins to proceed through their secretion pathway and to be secreted successfully. Three dimensional structure of Wnt proteins is stabilized by numerous disulphide bridges between 22 conserved cysteines and this structure together with the palmitoleic modification on serine are essential for the recognition of the receptor by Wnt protein (Janda et al., 2012)

7.1.2.2. Wnt secretion

Properly folded and modified Wnt molecules bind to the Wnt recycling cargo receptor Wntless/Eveness interrupted/Sprinter/Gpr177 (Bänziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009; Goodman et al., 2006) by their palmitoleate group. They are then transported from ER to Golgi apparatus and released from the receptor after acidification of the vesicular lumen (Coombs et al., 2010). After exocytosis, Wnt proteins can be passed to a number of extracellular carriers that can facilitate their spreading and transport to other cells.

7.1.3. Spreading and shaping of the Wnt gradient

Because the Wnts are hydrophobic due to their modifications with fatty acid moieties, they have only limited diffusion capabilities in the aqueous extracellular environment. Thus, without any additional help, Wnt proteins could not spread far and would often insert their acyl groups into lipid rafts (Zhai et al., 2004), concentrate on cell surface and act only on short distances as an autocrine (on the producing cell itself), juxtacrine (on neighbouring cells) or low range paracrine (on nearby cells) signal – and indeed, part of them does. However, the long range action of Wg in *Drosophila* wing disc has been estimated to be over 20 cell diameters (Zecca et al., 1996). This is made possible by extracellular carriers of Wnt proteins which conceal their hydrophobic surfaces.

7.1.3.1. Extracellular Wnt carriers

The solubility and spreading capabilities of Wnts can be dramatically enhanced by concealing the hydrophobic palmitic or palmitoleic acid moieties from the aqueous environment. Acyl group(s) can be inserted into the membrane or lipids or bound by hydrophobic pockets in proteins like that of the soluble Wg-interacting molecule (Swim) in *Drosophila*, which binds palmitic acid residue of Wingless (Mulligan et al., 2012), a *Drosophila* Wnt-1 homologue (Rijsewijk et al., 1987).

One way how to achieve long range signalling is to load Wnt proteins on extracellular particles generally called argosomes (Greco et al., 2001). For Wnts, the first discovered argosomes were lipoprotein particles in *Drosophila* called lipophorins (Panáková et al., 2005). Lipophorins are produced by the fat body and can bind Wnt proteins if endocytosed by Wnt producing cells or, alternatively, they shed Wnt proteins

from their surface, where Wnts accumulate. Later, exosomes (membranous vesicles derived from endosomes) were found to transport Wnt proteins bound to their cargo receptor Wntless (Gross et al., 2012; Korkut et al., 2009). Third, Wnts can be spread bound to heparan sulphate proteoglycans on the surface of migrating cells (Serralbo and Marcelle, 2014) or passed by heparan sulphate proteoglycans (HPSGs) from one cell to the next (Franch-Marro et al., 2005). Despite these options, Wnts remain predominantly short-range signalling molecules (Clevers and Nusse, 2012; Farin et al., 2016).

7.1.3.2. Secreted Wnt inhibitors

The Wnt gradient is further shaped by extracellular secreted Wnt inhibitors and by receptors of Wnt ligands on target cells themselves. The receptors on cell surface bind Wnt molecules and prevent them from further spreading (Baeg et al., 2004) as occurs on the *Drosophila* boundary between parasegments (section 7.2.2.2), where a single cell row on the anterior edge of one parasegment binds almost all Wnt protein Wingless (Wg) from the last row of cells from the preceding parasegment so that it effectively cut off the posterior Wnt gradient, which can then spread only to the front (Sanson et al., 1999). Secreted proteins block the binding of Wnts to their receptors on the level of the ligand or on that of a receptor. Soluble frizzled-related proteins (sFRPs) with a homology to the Wnt-binding cysteine-rich domain (CRD) of the Wnt receptor Frizzled (Dennis et al., 1999; Finch et al., 1997; Leyns et al., 1997; Rattner et al., 1997; Üren et al., 2000; Wang et al., 1997), Wnt inhibitory factor-1 (Hsieh et al., 1999) and multipotent inhibitors of Wnt, BMP/TGFβ/Nodal signalling from Cerberus/Dan family (Bell et al., 2003; Piccolo et al., 1999) bind Wnt proteins and block their binding to the receptors on target cells. However, they do not alter the Wnt proteins and can also protect them and facilitate their diffusion, as is the case of some sFRPs (Mii and Taira, 2009). On the other hand, the activity of the Wnt ligand can be permanently abolished by a cleavage of its N-terminus by a protease Tiki (Zhang et al., 2012) or by a removal of the palmitoleate group by an extracellular carboxylesterase Notum (Kakugawa et al., 2015; Zhang et al., 2015).

As a consequence of such complex regulation of Wnt spreading and activity, the range of Wnt signalling is an outcome of a combination of several factors and can be only approximated, but not accurately described by the expression of Wnt genes as sources of the Wnt signal.

7.1.4. Wnt/β-catenin signal transduction pathway

In the Wnt/β-catenin or so called canonical Wnt signalling pathway, transcription of target genes is regulated via changes in stability and abundance of β-catenin, a transcriptional co-activator of Tcf family transcription factors, through its phosphorylation-dependent ubiquitylation and degradation (Figure 1). Interestingly, β-catenin is also a cytoskeletal structural protein, that together with α-catenin mediates the interaction of actin cytoskeleton to cadherins in adhaerens junctions.

7.1.4.1. Inactive state

In the absence of a Wnt signal (Figure 1 – left), cytoplasmic β-catenin is targeted for degradation by multiprotein β-catenin destruction complex. The destruction complex is constituted by Axin (Behrens et al., 1998; Hart et al., 1998) and adenomatous polyposis coli (APC) (Munemitsu et al., 1995; Rubinfeld et al., 1993), which provide a scaffold for two kinases, Casein kinase-1α (CK-1α) and glycogen synthase-kinase-3β (GSK-3β) (Amit et al., 2002; Liu et al., 2002; Rubinfeld et al., 1996). β-catenin is first phosphorylated by CK-1α which primes it for further phosphorylation by GSK-3β. The latter phosphorylation creates docking sites for direct interaction with $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase that ubiquitylates β-catenin and targets it for degradation in proteasomes (Hart et al., 1999; Winston et al., 1999). Also YAP/TAZ proteins, otherwise mediators of Hippo signalling, are an integral part of the destruction complex and required for this process since they recruit β-TrCP (Azzolin et al., 2014). Phosphorylated β-catenin in turn bridges TAZ to β-TrCP, thereby targeting it for degradation (Azzolin et al., 2012).

In the OFF state of the pathway, β-catenin is thus rapidly degraded and kept in low levels in the cytoplasm and the nucleus. In its absence, Tcf/Lef transcription factors in the nucleus bind co-repressors, like those of CtBP (Brannon et al., 1999; Valenta et al., 2003) or Groucho/transducin-like enhancer of split [TLE, (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998)] families, which in turn recruit histone deacetylases (Arce et al., 2009; Billin et al., 2000; Chen et al., 1999). These silencing complexes reside on regulatory elements of Wnt target genes and block their transcription until the repression is alleviated by a Wnt signal.

7.1.4.2. Reception of a Wnt signal

Wnt ligands associate on the surface of target cells with extracellular cysteine rich domain of a seven-pass transmembrane G-protein coupled receptor from the Frizzled (Fz) family (Bhanot et al., 1996; Yang-Snyder et al., 1996) and mediates its interaction with various co-receptors. Combinations of distinct Frizzled paralogues with certain co-receptors result in selective activation of downstream signalling (Kikuchi et al., 2009) by recruiting different co-receptors (Verkaar and Zaman, 2010). The canonical Wnt/β-catenin pathway is triggered by a formation of complexes between Fz and LRP5/6 (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000) or Ryk co-receptor (Lu et al., 2004). Wnt grasps the CRD of Frizzled with two extended finger domains in an interaction which involves the palmitoleate moiety of Wnt (Janda et al., 2012) The binding of Wnt to LRP can be blocked by extracellular soluble Wnt competitive LRP5/6 ligands Dickkopf (Bafico et al., 2001; Semënov et al., 2001) or dual Wnt/BMP inhibitors Wise (Itasaki et al., 2003; Lintern et al., 2009) and Sclerostin (Li et al., 2005b). Human Dickkopf-1 is itself a target of Wnt/β-catenin pathway, which provides a negative feedback loop (González-Sancho et al., 2004). Also the co-receptors that trigger non-canonical pathways compete with LRP5/6 for Fz (Grumolato et al., 2010) thereby inhibiting canonical signalling.

Conversely, Wnt signalling can be potentiated by concurrent binding of secreted R-spondin proteins to their receptors LGR4/5/6 (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011) with RNF43/ZNRF3 E3 ubiquitin ligases as co-receptors (Hao et al., 2012; Chen et al., 2013; Zebisch et al., 2013). This limits the amount of available free RNF43/ZNRF3, which otherwise ubiquitylate Frizzled receptors and target it for endocytosis and degradation (Hao et al., 2012; Koo et al., 2012). Genes for R-spondin's receptors or co-receptors are also themselves targets of Wnt signalling (Barker et al., 2007; Hao et al., 2012; Van der Flier et al., 2007), providing positive or negative feedback loops, respectively, and *Lgr5* serving as a well characterized Wnt-dependent stem cell marker in the intestine (Barker et al., 2007) and many other organs and tissues [summarized in (Nusse and Clevers, 2017)].

7.1.4.3. The Wnt/β-catenin signal transduction cascade

According to most widely accepted current model of canonical Wnt pathway activation (Figure 1 – right), the Wnt-Fz-LRP5/6 ligand-receptor complex recruits the

cytoplasmic protein Dishevelled (Klingensmith et al., 1994; Noordermeer et al., 1994) via its interaction with Frizzled (Cong et al., 2004; Tauriello et al., 2012), which in turn brings to the LRP5/6 receptor Axin-GSK-3β complex (Zeng et al., 2008). This is facilitated by an association of Dishevelled with phosphatidylinositol kinases that produce PIP2, which brings more Axin-GSK-3β to the membrane (Pan et al., 2008) and by a formation of large signalosomes (Bilić et al., 2007) via polymerization of Dishevelled and Axin through their DIX/DAX domains (Kishida et al., 1999; Schwarz-Romond et al., 2007). GSK-3β then phosphorylates the cytoplasmic tail of LRP5/6 (Tamai et al., 2004) that primes it for further phosphorylation by CK1γ (Davidson et al., 2005; Zeng et al., 2005). Phosphorylated CK1γ motifs on LRP/6 tail directly block GSK-3β activity in the destruction complex (Cselenyi et al., 2008; Piao et al., 2008; Stamos et al., 2014).

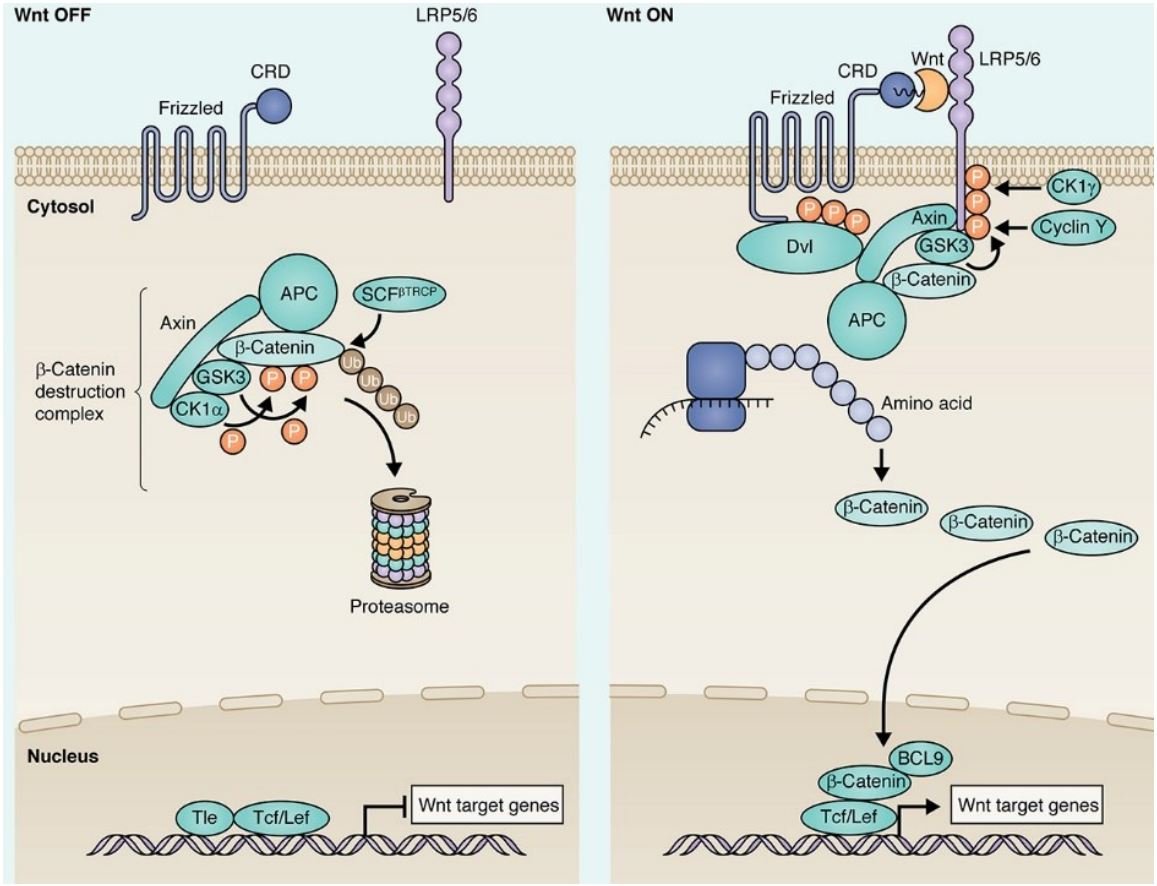


Figure 1 – Schematic representation of Wnt signalling pathway in inactive and active state

CRD = cysteine-rich domain of Frizzled receptor, Cyclin E= Cdk14-Cyclin E mitotic kinase complex, Tle = Groucho co-repressor

Reproduced from Steinhart and Angers (2018).

The activity of the Wnt/β-catenin pathway is also regulated independently by tankyrases that poly(ADP-ribosyl)ate the Axin of the β-catenin destruction complex and target it for ubiquitylation and degradation. Therefore, their inhibition leads to a stabilization of Axin and increased degradation of β-catenin (Huang et al., 2009).

Interestingly, besides being a signal transducer, β-catenin also has an important structural role in the cytoskeleton and cell adhesion, where it together with α-catenin mediates an interaction of actin to cadherins in adhaerens junctions (Jou et al., 1995). These two functions (signalling and structural) compete for the common pool of β-catenin (Hülsken et al., 1994)[1]. Assuming that the amount of β-catenin required for its cytoskeletal function remains more or less the same, changes in overall levels of β-catenin reflect the amount of nuclear β-catenin and thus the activity of Wnt pathway. From another point of view, changes in overall levels of β-catenin due to canonical Wnt signalling can affect also cell adhesion. Also other proteins of the destruction complex have an additional cytoskeletal functions. APC associates with plus ends of growing microtubules, stabilizes them and promotes their assembly (Mimori-Kiyosue et al., 2000; Munemitsu et al., 1994), an activity negatively regulated by the GSK-3β phosphorylation (Zumbrunn et al., 2001).

7.1.4.4. Tcf proteins and the control of transcription

As a result of the inactivation of the destruction complex, β-catenin is not phosphorylated, ubiquitylated and degraded. Instead, it can accumulate and translocate to the nucleus, where it binds to Tcf/Lef family transcription factors (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). The nuclear import happens thanks to the binding of β-catenin to Legless/BCL9 in complex with Pygopus (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), a transcriptional co-activator which carries the nuclear localization signal (Townsley et al., 2004). Nuclear β-catenin recruits UBR5 E3 ubiquitin ligase that drives ubiquitylation of Groucho/TLE co-repressor and its degradation (Flack et al., 2017) and/or by binding to a second low-affinity binding site displaces it from Tcf (Daniels and Weis, 2005) and mobilizes transcriptional co-activators p300 (Sun et al., 2000) and/or CARM1 (Koh et al., 2002) instead, which leads to de-repression and activation of transcription of Wnt target genes. Binding of β-catenin

[1] With the exception of *Caenorhabiditis elegans* which has two β-catenins with dedicated functions (Korswagen et al., 2000).

to Tcf and the association to p300 are disrupted by the binding of a small protein ICAT (Daniels and Weis, 2002; Tago et al., 2000).

Tcf has to compete for β-catenin with other transcription factors, e. g. FOXO (Hoogeboom et al., 2008). In the absence of phosphorylated β-catenin, YAP/TAZ also escapes the destruction complex and translocates to the nucleus where it regulates expression (Azzolin et al., 2014; Azzolin et al., 2012). These transcription factors thus mediate a proportion of Wnt response which is Tcf-independent and influence the outcome of Wnt signalling via cross talk with other signalling pathways (oxidative stress in the case of FOXO, Hippo signalling in the case of YAP/TAZ). Tcf proteins can act co-operatively with other transcription factors, for example PitX2, GATA3 [summarized by Archbold et al. (2012) or Cadigan and Waterman (2012)] or Sox (Kormish et al., 2010).

This general model would work in most of the situations when only one Tcf is present. In fact, whereas other bilaterians possess only one *Tcf* gene, with the exception of planarians (*Schmidtea mediterranea*) and trematodes (*Schistosoma mansoni*) of the phylum Platyhelminthes, whose genomes contain five or three *Tcf* genes, respectively (Archbold et al., 2012; Cadigan and Waterman, 2012), there are four different *Tcf* genes in vertebrates due to at least two rounds of whole genome duplication in the course of their evolution (Dehal and Boore, 2005). Since then, their Tcf proteins have specialized and diverged in function and today, each displays a slightly different mode of action after Wnt activation.

Vertebrate LEF1 (Travis et al., 1991; Waterman et al., 1991) functions exclusively as an activator of downstream genes (Liu et al., 2005), while TCF1 [encoded by *Tcf7* gene according to HUGO nomenclature, (van de Wetering et al., 1991)] is usually considered to be an activator (Liu et al., 2005) but has been reported to behave also as a repressor in some contexts (Roose et al., 1999; Standley et al., 2006). On the other hand, TCF3 [encoded by *Tcf7l1*, (Korinek et al., 1998)] functions as a pure repressor (Kim et al., 2000; Liu et al., 2005; Mašek et al., 2016; Merrill et al., 2004) and TCF 4 [encoded by *Tcf7l2*, (Korinek et al., 1998)] can exert both functions (Nguyen et al., 2009; Standley et al., 2006). *Drosophila* dTcf/Pangolin functions as a transcriptional repressor in the absence of Wingless signal by binding Groucho co-repressor (Cavallo et al., 1998).

These differences between Tcf proteins are given by their unequal affinities for co-repressors and co-activators due to presence or absence of their binding sites in Tcf sequence. Tcf proteins do not carry any such activity on their own and serve merely as a sequence-selective scaffold to build enhancer or silencer complexes on regulatory elements of Wnt target genes. For example, only TCF3 and some TCF4 variants (see further) possess

binding sites for CtBP1 co-repressor (Brannon et al., 1999; Valenta et al., 2003) and/or LVPQ/SXXSS motif which confers repressive capability (Liu et al., 2005). Besides binding Groucho co-repressor, *Drosophila* dTcf is further repressed via phosphoralytion by CBP, that is otherwise usually co-activator of other transcription factors (Waltzer and Bienz, 1998). In addition to de-repression/activation of a single Tcf, the phosphorylation by HIPK2 can cause an exchange of a Tcf on an enhancer for a different one after reception of Wnt signal in some cases, e. g. of the repressive TCF3 for the activating TCF1 (Hikasa and Sokol, 2011).

Tcf proteins interact with β-catenin via the Armadillo repeats of their N-terminal β-catenin binding domain (van de Wetering et al., 1997), whereas Groucho/TLE co-repressor binds in the central part to the GBS motif (Arce et al., 2009). On the C-terminus, there is a highly conserved HMG-box immediately followed by the basic tail, a stretch of basic amino acids which serves as a nuclear localization signal (Prieve et al., 1998). Together, they constitute the HMG DNA-binding domain (HMG DBD) (Travis et al., 1991; van de Wetering et al., 1991) that recognizes the specific Wnt responsive element CCTTTGATS (Hallikas et al., 2006; Korinek et al., 1997) in promoters of target genes. HMG-DBD functions as a monomer (Waterman et al., 1991) and forces a DNA bend (Love et al., 1995). Basic tail helps the HMG-box to bend the DNA, as shown in a closely related HMG DBD of the human SRY gene, (Li et al., 2006; Phillips et al., 2006). Alternatively, β-catenin can bind to SRY-related Sox transcription factors instead of Tcf. Sox proteins also contain HMG box, but trigger different transcriptional responses. They can thus compete with Tcf for β-catenin and modulate Wnt response (Zorn et al., 1999a). All invertebrate (with the exception of some platyhelminth *Tcf*s) and some vertebrate *Tcf* genes also encode for an accessory DNA binding domain. It is located C-terminally to HMG DBD and contains CRARF signature of amino acids (Hovanes et al., 2000) and several conserved cysteines, hence is called a C-clamp (Atcha et al., 2007). C-clamps are supposed to help HMG domains to select their binding sites by increasing the affinity of binding and restricting it only to certain sites (Chang et al., 2008). Consistent with this notion, some genes, like *Lef1* or *Cdx1*, are only activated by forms of Tcf that contain a C-clamp (Atcha et al., 2003; Hecht and Stemmler, 2003).

Not only there are four different *Tcf* genes in vertebrates, but they are subjected to alternative splicing that produces many isoforms (Duval et al., 2000; Hovanes et al., 2000; Van de Wetering et al., 1996) some of which are tissue specific and/or functionally distinct (Weise et al., 2010). For example, only E isoforms of vertebrate TCF1 and TCF4 possess a

full-length C-clamp (Atcha et al., 2003) which is necessary to bind and activate promoters of some genes (see above), while S isoforms contain a modified or truncated C-clamp and M isoforms lack the C-clamp (Weise et al., 2010).

Non-vertebrate metazoan animals do not possess multiple *Tcf* genes and when tested, Tcf/pangolin protein from a single species can rescue function only for a subset of vertebrate Tcf, but together they can cover them completely (Klingel et al., 2012). Although an alternative splicing and production of different Tcf isoforms from a single non-vertebrate *Tcf* gene seems to be plausible mechanism to compensate for the lack of gene diversity, it has not been extensively studied until recently. So far, only two splice variants of *Drosophila melanogaster* Pangolin (PanA and PanB), that differ in the second half of their HMG domains, have been described in the literature (van de Wetering et al., 1997). However, sequences of many splice variants from more protostome organisms are already available in on-line databases (unpublished observation), suggesting the presence of a broad repertoire of Tcf isoforms which mediate distinct developmental functions of Wnt/β-catenin signalling.

7.1.5. Wnt target genes

If we take a closer look at Wnt target genes, they can give us a hint of most important functions of Wnt/β-catenin signalling. A comprehensive (although not complete) overview of genes up- or down-regulated by canonical Wnt/β-catenin pathway can be found in the "Wnt target genes" section of "the Wnt homepage" (Nusse, 1997 - 2019). Hereafter, I select some of them that I consider most important and divide them to groups according to their developmental and cell physiological functions. Some of the roles of Wnt signalling suggested here by the genes regulated by Wnt/β-catenin pathway will be further discussed with their respective developmental processes or organ systems in the following chapters.

First, canonical Wnt signalling controls the expression for pro-proliferative genes *c-myc* (He et al., 1998), *n-Myc* (ten Berge et al., 2008a), *c-Jun* (Mann et al., 1999), cell cycle promoting genes *Cyclin D* (Shtutman et al., 1999; Tetsu and McCormick, 1999) or *CDC25* (Vijayakumar et al., 2011), the anti-apoptotic gene *Survivin* (Zhang et al., 2001) and the gene for the telomere maintenance enzyme *Telomerase* (Hoffmeyer et al., 2012), which points out to the positive role for Wnt/β-catenin signalling in the regulation of proliferation, progression through the cell cycle and cell and survival. Second, the control of Wnt over the transcription of the stem cell determinants *Oct4* (Cole et al., 2008), *Sox2* (Van Raay et al., 2005) and Tcf3 derepression of *Nanog* (Cole et al., 2008; Pereira et al., 2006) highlights the indispensable role of Wnt/β-catenin in the maintenance of stem cells.

A positive regulation of endoderm-inducing gene *Sox17* (Engert et al., 2013) and mesoderm master regulator *Brachyury* (Arnold et al., 2000; Yamaguchi et al., 1999) demonstrate the role of Wnt signalling in the specification of endomesoderm while the upregulation of *Snail* and *Fibronectin* (ten Berge et al., 2008b) with matrix metalloproteinases (Marchenko et al., 2002; Wu et al., 2007) along with a downregulation of *E-cadherin* (Huber et al., 1996; Jamora et al., 2003) is behind Wnt's ability to induce epithelial-mesenchymal transition and cell migration.

The control of Wnt/β-catenin signalling over the expression of *Neurogenin* (Hirabayashi et al., 2004) and *NeuroD* (Kuwabara et al., 2009) genes documents the ability of Wnt signalling to specify neural progenitors, whereas the regulation of *Nkx* (Lei et al., 2006), *Emx* (Theil et al., 2002) and other transcription factors is involved in the specification of neuron subtypes from the neuroectoderm, while the activation of *cdx* genes (Pilon et al., 2006; Pilon et al., 2007) in the anterior-posterior patterning of the gastrointestinal system and skeleton (endoderm and mesoderm, respectively).

7.1.5.1. Autoregulation of Wnt/β-catenin signalling

The robustness and exact level of Wnt/β-catenin signalling are partly achieved and stabilized by an involvement of autoregulatory feedback loops. These can be either direct, in which β-catenin and Tcf upregulate expression of some Wnt/β-catenin pathway's own component or regulator, or indirect, where a product of Wnt/β-catenin target gene might function as a transcriptional activator or a repressor of genes for Wnt pathway's components.

Not only Axin has a dual function as a member of the destruction complex and scaffold for LRP5/6 receptor phosphorylation and activation, but it is itself regulated by Wnt signalling. More precisely, of the two vertebrate *Axin* genes, *Axin2* is the target of Wnt/β-catenin pathway, whereas the other one, *Axin1*, is not (Jho et al., 2002; Lustig et al., 2002), although both are functionally equivalent *in vivo* (Chia and Costantini, 2005). Given that Axin represents the limiting component of the destruction complex (Lee et al., 2003), an increase in the amount of Axin thus provides a negative regulatory feedback loop for Wnt/β-catenin pathway. The degradation of β-catenin can be further elevated by a Wnt-dependent transcription of βTrCP, the E3 ubiquitin ligase that functions as a receptor for β-catenin ubiquitylation and degradation (Spiegelman et al., 2000). Another break against the pathway overactivation is represented by an upregulation of the Wnt competitive antagonist Dickkopf (González-Sancho et al., 2004; Chamorro et al., 2005; Niida et al., 2004) and the multivalent signalling antagonist Cerberus (Katoh and Katoh, 2006a) or the Tcf co-repressor TLE-3/Groucho (Kokabu et al., 2014).

On the other hand, Wnt/β-catenin signalling via LEF-1 induces expression of LEF-1 itself in a positive feedback loop (Filali et al., 2002; Hovanes et al., 2001), whereas a signalling via activating Tcf4 drives the expression of a repressor form of Tcf1 (Roose et al., 1999). Upregulation of *Frizzled* (Willert et al., 2002) enhance the signalling via an increase in the amount of available receptors, whereas Wnt proteins engage in an autocrine signalling in groups so distantly related as Vertebrata and Cnidaria (Deb et al., 2008; Kunz et al., 2004; Nakamura et al., 2011) to create positive feedback loops. In contrast, either the genes for *Drosophila* Wnt Wingless and its receptors Frizzled2 (Bhanot et al., 1996) and Arrow/LRP5/6 (Wehrli et al., 2000) are repressed in response to Wnt/β-catenin pathway activation to constitute a negative feedback (Cadigan et al., 1998; Yu et al., 1998). Also

Lgr5, the gene for the receptor of the Wnt coactivator R-spondin, is upregulated by Wnt/β-catenin signalling .(Barker et al., 2007)

Both positive and negative autoregulatory feedbacks thus can be present to precisely fine-tune the activity of β-catenin-dependent Wnt pathway and can make it either more resilient (in the case of negative feedback) or sensitive (for a positive feedback) to external disturbances.

7.1.5.2. Interactions of Wnt/β-catenin with other signalling pathways

Wnt signalling of course does not function in the organism alone, but in a regulatory landscape of other developmental signalling pathways. The crosstalk between Wnt signalling and these pathways then can be achieved directly by proteins of their signal transduction cascades or via transcription of target genes. Despite I do not intend to dissect the direct cross-talk in this place, I would like to briefly summarize the transcriptional control of other major signalling pathways by Wnt signalling, which alone may demonstrate their regulatory relationships during development, as it is a common feature that direct and indirect (via transcriptional regulation) crosstalk act in concert rather than against each other.

One of the hallmarks of early development is the opposition of Wnt and BMP during the specification of neural (Wnt) versus non-neural (BMP) body side (see further in the sections 7.2.1.3 and 7.2.3.1). This might be achieved by a transcriptional control of Wnt over the expression of the genes for secreted BMP antagonists Noggin (Hirsinger et al., 1997), Gremlin (Klapholz-Brown et al., 2007) and PRDC (Im et al., 2007) or by a reducing the transcription of the ligand BMP4 (Baker et al., 1999). In the context of mesoderm or the ridges of neural tube, coactivation of Wnt and BMP is required instead, which is achieved e. g. by a derepression of the BMP ligand Decapentaplegic in *Drosophila* visceral mesoderm (Yang et al., 2000) or in vertebrates by an upregulation of BMP4, as seen in colon cancer cells (Kim et al., 2002). Wnt/β-catenin pathway can also inhibit signalling by another member of TGF-β protein family Nodal/Activin by enhancing the expression of gene for its antagonist Follistatin (Willert et al., 2002; Yao et al., 2004). On the other hand, it directly activates the expression of yet another TGF-β member Nodal in the node (Granier et al., 2011; Rodríguez-Esteban et al., 2001) and of *Xenopus* Nodal-related protein 3 (*Xnr3*) in the Spemann's organizer of gastrulation (McKendry et al., 1997).

Via an upregulation of expression of the gene *Engrailed* (Danielian and McMahon, 1996; McGrew et al., 1999), which encodes a transcription factor, Wnt/β-catenin signalling can potentially stimulate the production of Hedgehog, as happens on the *Drosophila* inter-parasegmental boundary (Tabata et al., 1992) or during the patterning of imaginal (Zecca et al., 1995) and genital discs (Emerald and Roy, 1998). On the other hand, Wnt signalling upregulate the expression of *Gli3* that functions as a Hh inhibitor in the vertebrate neural tube (Alvarez-Medina et al., 2008).

Wnt/β-catenin signalling induces the expression of Notch ligands Delta-like 1 (Galceran et al., 2004; Hofmann et al., 2004) and Jagged1 (Estrach et al., 2006; Rodilla et al., 2009) to activate Notch signalling in neighbouring cells. This can result in an equilibrium with coactivation of both Wnt and Notch pathways in a population of Wnt expressing cells or by lateral inhibition to a mutually exclusive activity of either Notch or Wnt signalling. In insects, the production of Delta is directly positively regulated by Wg but then (with some delay) again indirectly downregulated in order to produce waves of Notch signalling activity during segmentation (section 7.2.2.1). It is possible that such dual regulation is present also in other phyla.

Genes for ligands from the fibroblast growth factor (FGF) family are often upregulated as targets of Wnt/β-catenin signalling (Hendrix et al., 2006; Chamorro et al., 2005; Kratochwil et al., 2002; Shimokawa et al., 2003). This suggests that canonical Wnt/β-catenin can activate FGF signalling.

Although the regulatory relationships may differ depending on a taxonomic group or particular paralogues involved, in general we can say that the Wnt/β-catenin signalling via the transcription of its target genes usually inhibits BMP signalling, stimulates Delta-Notch and FGF pathways, whereas it can both positively and negatively regulate Nodal signalling and positively regulates Hh in insects, but negatively in vertebrates. It is important to bear on mind that in some cases, influencing another signalling pathway may be responsible for some effects of Wnt/β-catenin pathway manipulations rather than direct regulation of gene expression. Taken together the whole complexity of Wnt signalling network, all the aspects of its regulation, the crosstalk and a broad spectrum of the Wnt transcriptional response, the effect of activation of Wnt/β-catenin pathway in a given time and space is difficult to interpret, because it is an outcome of a combinatorial action of multiple factors and can result in a wide repertoire of cellular responses.

7.2. Wnt/β-catenin signalling in metazoan development

Components of the Wnt pathway can be found in all metazoans from Ctenophora (comb jellyfish) (Pang et al., 2010), Porifera (Srivastava et al., 2010), Placozoa (Srivastava et al., 2008) and Cnidaria (Hobmayer et al., 2000; Putnam et al., 2007) at the base of the tree to all groups of Protostomia and Deuterostomia studied so far [see also Holstein (2012) and references therein]. Wnt signalling ability to instruct cell fate, orient cell division, its wide and conserved usage and ubiquitous occurrence in all metazoan lineages together with the absence of Wnt components from the genome of their closest unicellular relatives, Choanoflagellata (Fairclough et al., 2013; King et al., 2008), point to a key role of Wnt signalling in the origin of animal multicellularity and in the evolution of development in Metazoa (Loh et al., 2016). Below, I list main important developmental processes, in which the Wnt signalling plays an essential role across Metazoa.

The regulatory landscape of Wnt signalling is very complex and offers many modes of regulation. Thanks to this versatility, it is used in almost every aspect of metazoan development and in virtually every tissue and organ system. To cover all of them is beyond the scope of this text. Hereafter, I will thus focus only on the most pronounced features common to bilaterian body plans across all or most of the phyla and in which Wnt signalling plays an indispensable role – the determination of body axes, which is essential to understand the further involvement of Wnt signalling in following developmental processes, the segmentation of the body and the development of two major organ systems, that are common to all Metazoa, the central nervous system (CNS) and the digestive system. They were extensively studied in the past, yet the homology of these structures and the processes among the major bilaterian phylogenetic branches are still not completely resolved.

Wnt signalling takes part in the development of many other organ systems and morphological structures some of which might be of an ancient origin, while the others represent innovations of certain lineages and cannot be readily homologized to similar structures in other phyla. However, to describe the regulation of their development by Wnt signalling is beyond the scope of this text and was not studied in the course of this project.

7.2.1. Specification of body axes

Wnt/β-catenin is active along two perpendicular body axes during bilaterian embryonic development, which results in a bilateral body symmetry from which Bilateria derive their name. In combination with other signalling pathways like BMP, Wnt signalling therefore constitutes a system of coordinates which provides cells an information about their position in the embryo (Niehrs, 2010). There are more positions with distinct coordinates when two axes are present and cells hence can adopt a higher number of different developmental fates (Genikhovich and Technau, 2017), which enabled the evolution of more complex body plans in Bilateria. This system of coordinates represents a basis for the oriented specification and development of all major tissues and organs of the early embryo and is reflected also in their own polarity. A knowledge of the continuity with the role of Wnt/β-catenin signalling in the establishment of embryonic polarity is thus necessary for the proper understanding of Wnt functions in further development of these tissues.

7.2.1.1. Animal-vegetal axis and the specification of germ layers

Animal-vegetal (A-V) axis is the first established in development. The animal pole is determined by the position of polar bodies from oocyte divisions. Vegetal pole opposes the animal pole and often contains yolk and other maternally deposited determinants. The relationship of the A-V axis to the major body axes of larval or adult animals vary across species with a changing site and mode of gastrulation but as a rule of thumb, one can say that the animal pole usually corresponds to the anterior and develops into the head, whereas the vegetal pole coincides with the posterior (Martindale, 2005). Therefore, A-V axis can be most of the times identified with the anterior-posterior (A-P) axis. Both axes are hence often discussed also as a primary axis (Petersen and Reddien, 2009a), a term which covers both A-P and A-V axis. I will avoid the term primary axis in the present text to prevent a confusion between bilaterian axes and cnidarian oral-aboral axis, which is also sometimes called a primary axis (see further)

The A-V axis is tightly connected to the specification of germ layers. Cells that emanate from the vegetal pole give rise to the endoderm and mesoderm, while the cells at the animal pole to the ectoderm. The endomesodermal fate is specified via the regulation of *Brachyury*, *Cdx*, *Sox17*, *Nodal* (see above in the introduction to the chapter 7.1.5 and in

its section 7.1.5.2) and other endoderm and mesoderm master regulatory genes by maternal components of Wnt signalling present at the vegetal pole of the egg and later induced in vegetal cells. In organisms with the regulatory mode of development, the cell fate is determined by a position of the cell within the embryo with the positional information provided by extracellular molecular signals. The vegetal position is determined by a Wnt signal at the vegetal pole and all the cells in the vegetal part of the embryo adopt endo(meso)dermal fate. Components of Wnt signalling are localized to the vegetal pole for example in amphibians (Cui et al., 1996), fish (Lu et al., 2011), hemichordates (Darras et al., 2011) and sea urchins, in which they trigger canonical Wnt signalling that specifies endomesoderm (Croce et al., 2011; Logan et al., 1999; Smith et al., 2007; Wikramanayake et al., 2004).

In contrast, cell fates in organisms with a stereotypic (mosaic) development are determined by a segregation cell type-specific determinants during unequal asymmetric cell divisions, resulting in the gradual specialization of cell types in unique cell lineages. In this case, each individual cell division can be polarized along the A-V axis in a similar manner as is the whole embryo in organisms with regulative development. Intracellular Wnt determinants like β-catenin are asymmetrically stabilized localized to the vegetally positioned nuclei and degraded at the animal pole. This can happen early in development in all dividing cells along the entire A-V axis to polarize the vegetal daughter cells in each pair, or only in a specific round of cleavage to limit β-catenin to the most vegetal cells of the whole embryo (Petersen and Reddien, 2009a). In both modes, the β-catenin positive vegetal blastomeres give rise to (mes)endoderm but in the latter case not exclusively (Schneider and Bowerman, 2007). In nematodes, a Wnt signal produced by the vegetal-most blastomere P2 activates canonical Wnt pathway and stabilizes nuclear β-catenin in the vegetal daughter endodermal precursor E, but not in the mesodermal precursor MS during the division of the endomesodermal precursor EMS in order to segregate endoderm from the mesodermal lineage (Huang et al., 2007; Lin et al., 2009; Nakamura et al., 2005; Owraghi et al., 2010). Binary cell switch based on β-catenin stabilization in vegetal cell from each daughter blastomere pair is involved in the first two cell divisions in urochordates (Hudson et al., 2013) and in all cell division up to the establishment of bilateral symmetry in annelids (Schneider and Bowerman, 2007). In contrast, β-catenin becomes restricted to the vegetal cell population and subsequently to 4d mesenteloblast of the gastropod *Crepidula fornicata* (Henry et al., 2010) and the vegetal-most 4 cells contain stabilized β-

catenin and give rise to the endoderm in a nemertean *Cerebratulus lacteus* (Henry et al., 2008).

However, there is no clear-cut and both modes of development can occur in one organism. The mosaic development becomes more regulative as the development progresses and on the other hand, cells of an embryo with regulative development become more restricted reminiscent of a mosaic development. Under any circumstances, Wnt signalling confers vegetal cell identity and specifies endodermal fate. However, it should be noted that the orientation of a mitotic spindle in asymmetrical cell division is directly regulated by non-canonical Wnt planar cell polarity (PCP) pathway, but this happens in concert with the polarized activation of canonical Wnt/β-catenin signalling and localization of β-catenin in vegetally positioned nuclei. This is not restricted just to stereotypic development, but asymmetrical cell divisions regulated by non-canonical Wnt signalling play an important role in polarization of the embryonic axes also in the organisms with regulative development, like vertebrates (Gong et al., 2004).

7.2.1.2. Anterior-posterior axis

As stated above, the anterior-posterior (A-P) axis often originates from the A-V axis with posterior emerging from the vegetal pole. As a consequence of this relationship, an activated Wnt signalling is inherited in the posterior. The distinction from the A-V axis can come from the active inhibition of not only Wnts, but also other signalling pathways like BMP or Nodal in the anterior by a production of secreted Wnt inhibitors Dickkopf (Glinka et al., 1998) and sFRPs (Rattner et al., 1997) or by a multipotent inhibitors of Wnt and Nodal/BMP/TGF-β from the Dan family Cerberus (Piccolo et al., 1999), Coco (Bell et al., 2003) or others to enable the formation of head structures. When misexpressed, these antagonists are able to induce a formation of a new head. The situation with posterior Wnt signalling and anterior expression of Wnt inhibitors can be found in vertebrates (Blader et al., 1996; Chapman et al., 2004; Kemp et al., 2005; Leyns et al., 1997; Tendeng and Houart, 2006; Wang et al., 1997), urochordates (Imai et al., 2000; Lamy et al., 2006), cephalochordates (Yu et al., 2007), hemichordates (Darras et al., 2018; Pani et al., 2012), echinoderms (Kawai et al., 2016; Khadka et al., 2018), planarians (Petersen and Reddien, 2008), xenacoelomorphs (Martín-Durán et al., 2017), tapeworms (Jarero, 2018) and nematodes (Harterink et al., 2011). Wnts are expressed in the posterior growth zone of annelids (Bastin et al., 2015; Janssen et al., 2010), in which sFRP is expressed in the apical

organ (Marlow et al., 2014), arachnids (McGregor et al., 2008), crustaceans (Constantinou et al., 2016) and short-germband insects (Bolognesi et al., 2008), in which the Wnt gradient is opposed by its negative regulator Axin (Fu et al., 2012), similar to amphibians (Kofron et al., 2001). As a consequence, they have been recruited to establish also the A-P polarity of developing segments (see further) in segmented protostomes.

The paradigm of posterior Wnt activity and anterior Wnt inhibition is firmly established and the regulation of the A-P axis polarity by Wnt signalling has been extensively covered before (Petersen and Reddien, 2009a); however, many of the listed examples are based on the identification of A-P and A-V axes as a primary axis, do not include the information about anterior Wnt inhibitors and actually often represent the polarization of A-V axis. Less attention has been paid to downstream effector genes regulated by Wnt along the A-P axis. While the canonical Wnt/β-catenin pathway polarizes the A-P axis, directed cell divisions oriented by PCP pathway are responsible for orchestrated gastrulation movements and axis elongation by convergent extension (Gong et al., 2004; Steinmetz et al., 2007).

7.2.1.3. Dorsoventral axis and the specification of neural body side

In some protostomes, Wnts are produced in the ventral midline (Pruitt et al., 2014),which often originates by the fusion of the blastoporal lips (Nielsen et al., 2018). According to the theory of dorsoventral inversion (Arendt and Nübler-Jung, 1994; Nübler-Jung and Arendt, 1994), an inversion of D-V axis occurred in the chordate stem lineage and the dorsal midline of vertebrates, cephalochordates, urochordates and hemichordates is thus homologous to the ventral midline of insects, annelids and other protostomes.

Dorsal specification is tightly interconnected with A-V axis in anamniote vertebrates like *Xenopus* or zebrafish – after fertilization, Wnt determinants positioned maternally to the vegetal pole get along microtubule scaffold (in *Xenopus* oriented by a rotation of cortical cytoplasm) to the future dorsal side, where the activity of the canonical Wnt/β-catenin signalling specifies the dorsal endoderm (Lu et al., 2011; Miller et al., 1999; Rowning et al., 1997; Tao et al., 2005). What is usually called “dorsal” mesoderm and paradoxically requires inhibition of both Wnt and BMP (Carron and Shi, 2016) is in fact anterior tissue, which gives rise to the head (Arendt and Nübler-Jung, 1997). True dorsal mesoderm is specified by Wnt signalling in combination with Activin (Nasevicius et al., 1998; Sokol and Melton, 1992) and Wnt inhibition is only required later for the

differentiation of notochord (Hoppler and Moon, 1998; Reintsch et al., 2005). Embryonic source of Wnts in amniotes besides the posterior node lies also in the primitive streak (Liu et al., 1999; Mohamed et al., 2004) that corresponds to the dorsal midline and by a posterior migration of the (Hensen's) node also reminds closing blastopore lips (Arendt and Nübler-Jung, 1997). Wnt signalling is seemingly not involved in the specification of D-V axis in cephalochordates but it is still active dorsally (Holland et al., 2005).

Later, midline Wnts signal to the neighbouring tissue or the β-catenin positive cells sort to a position along the midline, so the nuclear β-catenin and Wnt pathway activity can be observed in the paraxial mesoderm (somites in vertebrates) (Lauri et al., 2014; Reintsch et al., 2005) and the medial neuroectoderm in protostomes (Demilly et al., 2013)/the lateral neural plate border/dorsal neural tube in chordates (García-Castro et al., 2002; Megason and McMahon, 2002), but not in the midline (axochord/notochord, medial neural plate/ventral neural tube) itself (Holland et al., 2005; Lauri et al., 2014). In chordates, this is achieved rather by moving the *Wnt* expression from the midline to lateral domain due to a mutual inhibition of Wnt/β-catenin signalling with Hh in (see further in the Discussion, section 11.2.2.2). Wnt signalling induces expression of BMP inhibitors, which restricts BMP2/4 expression ventrally (see sections 7.1.5.2 and 7.2.3.1 for citations). This results in the formation of two opposing, D-V oriented Wnt and BMP gradients (Lichtneckert and Reichert, 2005). The reversion of these gradients in chordates relative to other bilaterians and – as a consequence – of a position of central nervous system, contractile vessel(s)/heart, notochord or its homologues supports the theory of dorsoventral inversion. For a continuation and further details see the section 7.2.3.1 about the early development of the nervous system. In general, Wnt signalling specifies neural body side (dorsal in chordates, ventral in the rest of Bilateria) and BMP non-neural body side (ventral in chordates and dorsal in other bilaterians).

7.2.2. Segmentation

Segmentation, i. e. a modular organization of a body from repetitive units called segments (Hannibal and Patel, 2013), is a pronounced feature of animal body plans, which led in the past to a grouping of annelids and arthropods into the paraphyletic group "Articulata". New molecular phylogenies broke this cluster (Aguinaldo et al., 1997; Dunn et al., 2014; Telford et al., 2015) and undisputable segmental body plans thus can be found in all three major clades of Bilateria: in Panarthropoda (insects, crustaceans, myriapods, diplopods, chelicerates, onychophorans and tardigrades) from the clade Ecdysozoa and in Annelida from the clade Spiralia of Protostomia, and in Chordata (vertebrates, urochordates, cephalochordates) from the clade Deuterostomia, but also in some smaller groups, e. g. Cestoda (tapeworms) within the phylum Platyhelminthes from Spiralia. However, there are many serially repetitive (metameric) structures reminiscent of segments (and for the distinction called metameres or pseudosegments) in most of the other animal phyla (Hannibal and Patel, 2013). Nevertheless, it is questionable, whether the segments of different groups are homologous and the last common ancestor (LCA) of these lineages was segmented or not.

The segmentation process has four chronologically overlapping stages: segment specification, establishment of the segment polarity, segment formation and the specification of the segment identity. I will cover them in the following sections.

7.2.2.1. Segment specification

In vertebrates, sequentially segmenting arthropods (incl. short-germband insects – e. g. *Tribolium*) and the secondary (post-metamorphic) segmentation of annelids, new segments are being added sequentially during the growth in development (Peel et al., 2005). This happens in the segment addition (or posterior growth) zone (SAZ or PGZ, respectively), a proliferative region between the last body segment and the posterior terminus (the pygidium in arthropods and annelids). Since the canonical Wnt/β-catenin signalling is active in the posterior where it specifies the A-P axis (cf. chapter 7.2.1.2), it is naturally present in the PGZ in either vertebrates and invertebrates (Martin and Kimelman, 2009). The generation of segments is ensured by a constitutive proliferation in SAZ with a concomitant cyclic activation of differentiation genes by a signalling pathway oscillator of the segmentation clock mechanism. The segmentation clock is a signalling/gene regulatory

network (GRN) which through feedback regulation generates waves of gene expression in the progenitor cells of the SAZ. Newly formed rows of cells thus inherit a periodically repetitive gene expression fingerprint. In vertebrates, the oscillator involves periodic activation of Delta-Notch and FGF pathways with an out-of-phase waves of Wnt/β-catenin signalling (Aulehla et al., 2003; Pourquié, 2011). The formation of new posterior segments in arachnids (Stollewerk et al., 2003) and hemimetabolous insects (Pueyo et al., 2008) requires a production of cyclic segmental stripes of gene expression by Delta-Notch pathway. Delta-Notch activation happens probably through Wnt/β-catenin signalling that drives the expression of *Delta* and *Caudal* (an orthologue of vertebrate *Cdx* genes) (McGregor et al., 2008). This in turn potentiates Wnt and hence more *Caudal* expression, but at the same time Caudal represses *Delta*, which creates the oscillations (Chesebro et al., 2013). The mechanism involving Delta-Notch pathway has been lost and only the activation of *Caudal* by Wnt/β-catenin signalling was retained in short-germband holometabolous insects (Bolognesi et al., 2008). Wnt signalling (Janssen et al., 2010) and Notch signalling (Rivera and Weisblat, 2009) are also present in the annelid SAZ, which suggests that the growth zones, and possibly also the segmentation mechanisms are homologous.

The segments of long-germband insects like *Drosophila* and the so called primary (larval) segments of annelids are specified (simultaneously or sequentially) by a division of the trunk without the growth. The segmental expression of genes is achieved by mutual interactions of maternally positioned factors and their downstream pair-rule genes that can (in the case of *Drosophila*) freely diffuse through the syncytial blastoderm (Akam, 1987; Peel et al., 2005) or are segregated to certain blastomeres during the embryonic cleavage (as in annelids). However, pair-rule genes are themselves targets of Wnt signalling in short-germband insects (Oberhofer et al., 2014) and in either case the result is a formation of a segmental pattern of Wnt expression and other genes that specify the boundaries of first embryonic segments. Therefore, Wnt/β-catenin signalling is involved in the specification of both types of segments in all three major segmented animal phyla, which points to its ancient role in the segmentation process.

7.2.2.2. Establishment of the segment polarity

The posterior Wnt activity is inherited by the segments themselves in their own anterior-posterior (A-P) polarity and sets their boundaries. However, first embryonic segments do not correspond to the final segments in insects, hence are called parasegments.

The mechanisms of molecular patterning of (para)segments are best known in *Drosophila*: the canonical Wnt Wingless (Wg) produced by the posterior-most row of cells in each parasegment maintains the expression of *Engrailed* in the neighbouring anterior-most row of cells of the following parasegment (Bejsovec and Martinez Arias, 1991; DiNardo et al., 1988; Heemskerk et al., 1991). *Engrailed* then drives the production of Hedgehog (Tabata et al., 1992), but promotes by GSK-3β and CKI phosphorylation the degradation of Cubitus interruptus (Jia et al., 2002; Price and Kalderon, 2002) and represses its transcription (Eaton and Kornberg, 1990) in order to inhibit the transduction of Hh signalling and prevent the Hh producing cells to respond themselves to Hh. The secreted Hh protein via Patched receptors on the Wg producing cells and the stabilization of the transcription factor Cubitus interruptus/Gli in turn stimulates the production of more Wg (Ingham, 1993; Ingham and Hidalgo, 1993; Von Ohlen and Hooper, 1997). Patched at the same time prevents the majority of parasegment except the anterior En positive cells and including the Wg producing cells, to respond to Wg by the expression of *En* (DiNardo et al., 1988; Ingham et al., 1991). The binding of signalling molecules to their receptors also cut off the following rows of cells from the signal., so the signal can freely spread only to the other side, towards the middle of the segment (Gritzan et al., 1999; Sanson et al., 1999). The positive feedback loop between Wg and Hh producing cells results in a formation of sharp boundaries between segments and opposing anterior Hh and posterior Wg gradients.

In short-germband insects, like the beetle *Tribolium castaneum*, the sequential addition of segments represents a more ancestral state of the segmentation process. Wg in the growth zone activates Hh and these two pathways drive transcription of virtually non-overlapping sets of genes (Oberhofer et al., 2014), consistent with their mutually exclusive function within a positive feedback loop in the establishment of (para)segment polarity in *Drosophila*. Indeed, the same mechanism seems to be conserved also in the short-germband insects (Farzana and Brown, 2008). This regulatory pattern is connected to growth in SAZ and thus naturally inherited by each forming segment due to periodic fluctuations in Wnt signalling caused by the segmentation clock (see the previous section).

Despite the annelid growth zone is unaffected by cyclopamine inhibition of Hh signalling and Hh is thus probably (unlike Wnts) not involved in segment formation but only in segment polarity, the same mechanism of A-P segment polarity seems to be also in place in annelids (Dray et al., 2010) which places the origin of this segmentation mechanism at least to the last common ancestor of all Protostomia.

Hedgehog signalling does not take part in the oscillator of vertebrates. Nevertheless, as mentioned before the Wnt/β-catenin is involved (Aulehla et al., 2003) and *Engrailed* is expressed in the posterior of somites in cephalochordates (Holland et al., 1997). This raises the possibility that both segmentation mechanisms (deuterostomous and protostomous) are elaborations of an ancestral common segmentation mechanism which involved Wnt/β-catenin signalling and the role of Hh signalling has been lost in vertebrates or it represents an innovation of the protostomous lineage.

7.2.2.3. Segment formation

During the development of long-germband insects (e g. *Drosophila*), molecular events initially divide the blastoderm to repetitive fields with the same expression pattern with the sharp boundary between Wnt/Ptch and Hh/En positive cells, the parasegments, as described above. However, the morphological boundary between segments, which compartmentalize the embryo, is positioned elsewhere. Segmental grooves are originate by apical constriction of cells at a posterior border of *En* expression and their formation is dependent on the presence of En and Hh signalling and repressed by canonical Wg signalling (Larsen et al., 2003). The final segment is thus made up by majority from a rear part of the anterior parasegment and from the frontal part of the following parasegment. This happens only in the ectoderm but not in the mesoderm (Lawrence et al., 1985). As a result, the muscles are in a parasegmental position, out of register with the segmented cuticle, which ensures that they have contact with two neighbouring segments and can rotate them around their articulated joint on the principle of a lever. Insect appendages arise at the parasegmental boundary and are thus positioned within the final segment with the musculature from both parasegments. An analogical situation can be found in vertebrates in which the sclerotomes of somites redistribute to form the posterior part of one and the anterior part of the following vertebra (Aoyama and Asamoto, 2000). However, myotomes stay in the position of somites, so that the intervertebral muscles are out of register with vertebrae of the vertebral column for the same mechanistic reasons. Unlike in these two groups, no such redistribution of embryonic segments was observed in annelids, in which the morphological boundaries arise between the stripes of *Wnt* and *En* expression and thus coincide with the boundaries between parasegments (Dray et al., 2010). It thus appears that Engrailed might not be the key regulator of the septum formation and compartmentalization, but its ancestral function rather could be in the specification of

neurons (Patel et al., 1989). Consequently annelid parapodia arise only from one embryonic segment and most of their muscles are intrasegmental which corresponds to the function of annelid segments as predominantly hydrostatic compartments (Nielsen, 2012). although it is also expressed segmentally in annelids (Prud'homme et al., 2003) and chordates . Hence the segments of annelids seem to be homologous to parasegments of insects and to early somites (before the redistribution of sclerotome) of chordates.

7.2.2.4. Specification of the segment identity

Finally, individual segments differ in their position along the A-P body axis. They often bear body outgrowths and appendages that are often specialized and differ by their function according to their position in the body. The identity of segment or even of a specific region in the body of an unsegmented animal is determined in development by a certain combination of homeotic (*Hox*) gene expression in the given region, the *Hox* code, which is conserved among chordates, arthropods (Duboule and Dollé, 1989) and annelids (Kourakis et al., 1997). *Hox* genes are expressed in an overlapping staggered anterior-posterior sequence with a spatiotemporal co-linearity (i. e. the *Hox* genes are expressed spatially and temporally in the same order in which they are located on the chromosome) (Duboule and Morata, 1994; Iimura and Pourquié, 2007). In vertebrates, the expression of *Hox* genes is regulated by a concerted action of posterior to anterior gradients of retinoic acid (RA), FGF and Wnt signalling molecules (Bel-Vialar et al., 2002; Dubrulle et al., 2001; Ikeya and Takada, 2001; In der Rieden et al., 2010; Liu et al., 2001; Nordström et al., 2006; Shimizu et al., 2006; Schubert et al., 2006). It seems that an early Wnt program initiates a late FGF- and RA-based program (Nordström et al., 2006) and the regulation of *Hox* genes can be either direct or mediated by *Cdx* genes (Faas and Isaacs, 2009; Ikeya and Takada, 2001; Isaacs et al., 1998; Shimizu et al., 2006) from the *ParaHox* cluster, the evolutionary sister of the *Hox* cluster (Brooke et al., 1998). The nested or overlapping sequential expression of *Hox* genes is then given by their differential responsiveness to these signals and from their cross-regulation (Gould et al., 1997). In *Drosophila*, expression of *Hox* genes in each segment is selectively triggered by combinatorial inputs of a segmentation GRN (which is highly derived even compared to the ancestral insect state) and by their mutual regulations (Miller et al., 2001). The regulation of *Hox* genes outside of these species has not been systematically studied but the regulation by retinoic acid has been so far observed only in chordates. On the other hand, there are known cases, when *Hox* genes are regulated

also by Wnt/β-catenin signalling in both chordate (In der Rieden et al., 2010; Nordström et al., 2006) and non-chordate animals, e. g. the nematode *Caenorhabditis elegans* (Eisenmann et al., 1998; Korswagen et al., 2000; Streit et al., 2002; Teng et al., 2004) or the fruit fly *Drosophila melanogaster* (Riese et al., 1997). Wnt/β-catenin signalling also positively regulates either *Fgf* and *Cdx/caudal* (cf. the introduction to the chapter 7.1.5 and its section 7.1.5.2) and is involved in the establishment of the A-P polarity from the early development (section 7.2.1.2). It is plausible that some of its later effects are mediated by *Hox* genes. Therefore, it might be worth of testing, whether some *Hox* genes in other species can respond to the A-P Wnt gradient in a similar manner as they do to the RA gradient in chordates.

Although some organ systems in otherwise segmented organisms are not segmented morphologically, they still carry the legacy of the segmentation in the expression of segment identity genes. It is translated to a differentiation of distinct cell types according to their position along the A-P axis and hence functional segmentation of the tissue, e. g. the nervous system or the digestive tube (sections 7.2.3.3 and 7.2.4.2).

7.2.3. Central nervous system

In general, developing tissues and organs follow similar rules as segments: first, the tissue has to be specified. Second, the organ primordia acquire polarity and third, the tissue is patterned along the polarizing gradients. Because of the different gene/protein nomenclature in chordates and protostomes, it will be the best to describe the process of neural specification and patterning on the example of vertebrate nervous system and then translate the homologous, evolutionarily conserved processes into the context of protostomes.

7.2.3.1. Specification of neuroectoderm

The formation of neurogenic ectoderm (or the neural plate) is achieved by the inhibition of BMP signalling on the dorsal body side in the process of neural induction, which results in a neurogenic region on the dorsal (in vertebrates, ventral in others) body side (Ozair et al., 2013; Stern, 2005). This is achieved by a dorsal production of several soluble signalling molecules from the transforming growth factor (TGF)-β family – Chordin (Sasai et al., 1995), Noggin (Lamb et al., 1993) and Follistatin (Hemmati-Brivanlou et al., 1994). Therefore, the specification of neuroectoderm is tightly coupled to the D-V axis specification of the early embryo (cf. the section 7.2.1.3). Noggin and Follistatin are direct targets of Wnt signalling (see the section 7.1.5.2) and hence are secreted by the cells of the organizer of gastrulation and its derivative, the primitive streak (i. e. the dorsal midline). These secreted antagonists block the epidermal-inducing activity of BMP (Wilson and Hemmati-Brivanlou, 1995) and unlock the neural fate of ectodermal cells. On the other hand, *Chordin* is not direct Wnt target, but it is a target of Nodal-related proteins instead (Ramis et al., 2007; Wessely et al., 2004) and of transcription factors Twin and Siamois (Reid et al., 2012). The early phase of neural induction hence probably involves an activation of *Chordin* expression by Nodal/Nodal-related/Activin signalling from the organizer/dorsal mesoderm (Le Petillon et al., 2017). Nevertheless, Nodal and Nodal-related proteins are themselves direct transcriptional targets of Wnt/β-catenin pathway (see above in the section 7.1.5.2) and maternal or extraembryonic Wnts probably trigger Nodal signalling early on in the organizer (Shen, 2007; Turner et al., 2016). Also Siamois and Twin are effectors and direct transcriptional targets of Wnt/β-catenin signalling (Brannon et al., 1997; Brannon and Kimelman, 1996; Carnac et al., 1996; Laurent et al., 1997). Wnt/β-catenin and Nodal signalling thus act synergistically on the activation

of *Chordin* (Reid et al., 2012), which responses to changes in the canonical Wnt signalling activity as its indirect target (Wessely et al., 2004). Canonical Wnt/β-catenin signalling also directly activates transcription of proneural genes, like *Neurogenin* or *NeuroD* and pro-proliferative and cell cycle promoting genes (see the chapter 7.1.5) and hence drives neurogenesis. Note that the first role of Wnt/β-catenin signalling in development is the specification of the AV axis and of mesendoderm (see the section 7.2.1.1). We can thus conclude that the Wnt/β-catenin signalling can be traced as an ultimate cause of nervous system development, but it directs cells towards the neural fate only in the ectodermal context, after the germ layers had been established.

In protostomes, like *Drosophila* or *Tribolium*, the homologues of BMP and Chordin are named Decapentaplegic (Dpp) and Short of gastrulation (Sog), respectively (Biehs et al., 1996; Holley et al., 1995; Zee et al., 2006). They are activated differently than in vertebrates [via ventral nuclear import of NF-κB protein Dorsal, differential sensitivity and cross-regulations of its target genes (Nunes da Fonseca et al., 2008; Stathopoulos and Levine, 2002)] and the future mesoderm that performs the neural induction (via the regulation of Sog by Dorsal) is located on the ventral surface, so the neuroectoderm differentiates in two longitudinal stripes between the mesoderm and epidermis. The mesoderm is later internalized and the neuroectoderm fuses to form a new ventral midline (Klämbt et al., 1991). Despite these differences, the mechanism of neuroectoderm specification is otherwise very similar. Dpp induces dorsal and epidermal fates (Wharton et al., 1993) and its inhibition by Sog is necessary for the ectoderm to assume neural fate (Ferguson and Anderson, 1992) with segmental Wnt expression in the neurogenic regions of the central nervous system (Chu-LaGraff and Doe, 1993; Russell et al., 1992). The system of neural specification hence appears to be conserved and evolutionarily ancient, dating back to the last common bilaterian ancestor.

7.2.3.2. D-V/medio-lateral patterning of the neural tube/plate

In chordates, components of canonical Wnt pathway are present in the primitive streak, i. e. the dorsal midline during gastrulation (Hume and Dodd, 1993; Chapman et al., 2004; Nakaya et al., 2005), but unlike in other Bilateria, the Wnt/β-catenin signalling later becomes inhibited in the midline, persists only in lateral domains and is replaced by Sonic hedgehog (Shh) produced by the newly formed chorda dorsalis and the prechordal plate mesoderm (Marti et al., 1995; Roelink et al., 1994; Shimeld, 1999). In a process of neurulation, which is specific to Chordata and Hemichordata (Miyamoto and Wada, 2013)

and requires Wnt signalling and BMP signalling (Steventon et al., 2009; Ybot-Gonzalez et al., 2007), the Wnt-positive neural border (a non-neural ectoderm) forms ridges on both sides of the neural plate, the neural folds, that close together and internalize the neuroectoderm as the neural tube (Nikolopoulou et al., 2017). During this process, some cells from the edge of the closing neural tube undergo epithelial-mesenchymal transition and populate the body as the neural crest cells. Whereas the neural crest induction requires canonical Wnt/β-catenin signalling (García-Castro et al., 2002; Leung et al., 2016), the migration of neural crest cells depends on non-canonical Wnt pathways (Carmona-Fontaine et al., 2008) and the activation of Wnt/β-catenin pathway promotes pigment cell formation (Dorsky et al., 1998).

Wnt proteins are then produced by the new dorsal midline formed from the fused neural ridges of the neural tube, where they stimulate the proliferation of neural progenitors (Chesnutt et al., 2004; Megason and McMahon, 2002). The dorsal neural midline produces BMP and Wnt proteins (Liem et al., 1997; Muroyama et al., 2002) whereas the ventral neural midline and the nearby chorda Sonic hedgehog (Shh) (Echelard et al., 1993; Krauss et al., 1993; Marti et al., 1995; Roelink et al., 1994) whose dorsal limit regulated by a ventral expression of *FoxA* genes (Mavromatakis et al., 2011). These opposing signals pattern the neural tube along the D-V axis into several functional domains, as the neural progenitors differentiate acquire the expression profile with distinct combinations of transcription factors according to the combination of signals they receive (Le Dréau and Martí, 2012; Liem et al., 2000). Increasing concentration of Shh and decreasing Wnt and/or BMP signals promote neuronal differentiation to progressively more ventral (motor neuron) neural fate (Ericson et al., 1995; Roelink et al., 1994) and to glial floor plate (Echelard et al., 1993; Ribes et al., 2010; Roelink et al., 1994), while higher Wnt and BMP and lower Shh activity specify a more dorsal fate (Li et al., 2009; Liem et al., 2000; Muroyama et al., 2002). As a consequence, the neural tube is subdivided to a dorso-ventral series of distinct molecular territories with specific combinations of expression of transcription factors, which results in the differentiation of longitudinal columns of the typical functional types of neurons in the spinal cord (Dessaud et al., 2008; Le Dréau and Martí, 2012; Wilson and Maden, 2005). The D-V stratification is achieved via differential activation and cross-regulation of these neuronal differentiation transcription factors by the aforementioned polarizing events. Among the most prominent is the *Nk*/*Pax* system in neural progenitors around the neural canal. The genes for Nk and Pax transcription factors are expressed in *Nk*/*Pax* non-overlapping pairs, in which *Nk* gene has ventral affinity and is nearly abutted by the

expression of a corresponding *Pax* gene with a dorsal affinity. This results in several domains with unique *Nk*/*Pax* code. In the first pair, ventral *Nk2.1* (brain) or *Nk2.2* (spinal cord) specify motoneurons and abut dorsal *Pax6* that specifies predominantly interneuron progenitors (Briscoe et al., 1999; Ericson et al., 1997; Stoykova et al., 2000). In the second *Nk*/*Pax* pair, ventral *Nk6.1+Nk6.2* does overlaps *Pax6*, but does not reach the dorsal *Pax3* and *Pax7* (Briscoe et al., 2000; Li et al., 2005a). The ventral and dorsal affinity of *Nk* and *Pax* genes, respectively, is thought to result from the fact, that *Nk* genes are positively regulated by ventral Shh, whereas *Pax* genes negatively regulated by Shh and positively by Wnt (Ericson et al., 1997; Guner and Karlstrom, 2007; Le Dréau and Martí, 2012). In contrast, *Pax2*, *Pax5* and *Pax8* are expressed broadly in differentiating neurons of the intermediate zone in the hindbrain and spinal cord (Asano and Gruss, 1992; Nornes et al., 1990; Plachov et al., 1990) and to my knowledge are not mirrored by any particular *Nk* gene.

Despite it might be also internalized, the neuroectoderm is not folded into neural tube in protostomes because the neurulation does not take place. Their nervous system can be imagined as a chordate neural tube, but spread into a single plane. Therefore, the mediolateral pattern of transcription factors and differentiating neuronal subtypes is not converted into dorso-ventral and the medial and lateral positions in the central nervous systems of protostomes correspond to the ventral and dorsal in chordates, respectively. Nevertheless, except the missing neurogenic function of *Pax6* in *Drosophila* (and perhaps other insects), the overall molecular topography of the medio-lateral/dorso-ventral patterning of the neuroectoderm in protostomes bears many similarities to that observed in chordates, including the *Nk*/*Pax* system [reviewed by (Arendt et al., 2008; Arendt and Nubler-Jung, 1999)]. This points out to the ancient and common origin of central nervous system or at least its patterning mechanism in the bilaterian and even pre-bilaterian ancestors (Arendt et al., 2015), although this view has been rivalled recently (Martín-Durán et al., 2017). However, as to my knowledge, Wnt signalling is not involved in the mediolateral patterning of the trunk nervous system in *Drosophila*.

7.2.3.3. The A-P patterning of the central nervous system

The rostro-caudal patterning of the neural plate/neural tube is regulated by posterior-to-anterior gradients of Wnt/β-catenin signalling, e. g. via Wnt3a (Kiecker and Niehrs, 2001; McGrew et al., 1995; Nordström et al., 2002), retinoic acid and Fgf signalling

which result in a staggered sequence of *Wnt*, *Hox* and *ParaHox* genes and other homeobox-containing transcription factors (Figdor and Stern, 1993; Shimizu et al., 2006). In the A-P patterning of neuroectoderm (and other tissues), Wnt/β-catenin signalling in general promotes caudal (posterior) characteristics. The regulation of *Hox* genes was discussed before (see the section 7.2.2.4) and the subdivision of neuroectoderm by *Hox* or *ParaHox-Hox* code is present across vertebrates (Liu et al., 2001; Shimizu et al., 2006), arthropods and annelids (Steinmetz et al., 2011). There are also other transcription factors that divide the nervous system into larger functional territories, mostly anterior to *Hox* expression. Among the most notable of these is the subdivision of the brain to the frontal *otx*- and a posterior *gbx*-expressing domains separated by a sharp boundary, the midbrain-hindbrain boundary (MHB), also called the isthmic organizer (Rhinn and Brand, 2001). At first, canonical signalling via Wnt8 posteriorizes the neuroectoderm and determines the position of the MHB by driving *Gbx* expression (Rhinn et al., 2005). The boundary is then stabilized by mutual inhibition of *Otx* and *Gbx* expression (Li and Joyner, 2001) and by Wnt1, which is engaged in a positive feedback loop with FGF8 (Canning et al., 2007) and promotes the expression of the transcription factors *Engrailed* (Danielian and McMahon, 1996) and perhaps (indirectly via Engrailed) also of *Pax2/5/8* (Canning et al., 2007; Liu and Joyner, 2001). Besides the patterning and stabilization of the boundary, the ultimate role of Wnt/β-catenin signalling in the MHB is to promote cell proliferation of neural precursors (Panhuysen et al., 2004) and to specify dopaminergic neurons in the midbrain (Arenas, 2014; Castelo-Branco et al., 2010; Castelo-Branco et al., 2004; Castelo-Branco et al., 2003). Based on the molecular fingerprint, the isthmic organizer has been implied to exist in Hemichordata (Pani et al., 2012) and Arthropoda (Hirth et al., 2003; Urbach, 2007). The A-P subdivision of the neuroectoderm to the anterior *Otx*-expressing and posterior *Gbx*-expressing part is also present in annelids (Steinmetz et al., 2011); however, the isthmic organizer in annelids has not been documented so far.

The midbrain differentiation requires *Pax2/5*, *En* (Schwarz et al., 1999) and *Nk2.1* (Kimura et al., 1996) but expresses also *Otx* (Boncinelli et al., 1993). It contains another organizing signalling centre, the zona limitans intrathalamica (ZLI) which is located on the interface between the anterior *Fezf* and the posterior *Irx* domains (Irimia et al., 2010), expresses *Hedgehog* and *FoxA* (Britto et al., 2002) and its homologue was identified besides vertebrates also in hemichordates (Pani et al., 2012). The *Fezf-Irx* boundary is present also in cephalochordates (but not in hemichordates) and *Drosophila* (Irimia et al., 2010), but a ZLI homologue in Spiralia is not known.

The differentiation of the rostral (telencephalic) part of CNS and of the eyes is determined by a Groucho-dependent repression of Wnt production by the transcription factor *Six3* (Kobayashi et al., 2001; Lagutin et al., 2003) and of Wnt target gene repression by Tcf3 (Kim et al., 2000; Mašek et al., 2016), dependent on β-catenin degradation by a destruction complex with Axin and GSK-3β (Heisenberg et al., 2001; van de Water et al., 2001). The forebrain (telencephalon and diencephalon) is then characterized by an expression of *Pax6* (Georgala et al., 2011; Schwarz et al., 1999; Stoykova and Gruss, 1994) ventrally abutting to *Nk2.1* (Sussel et al., 1999) (similar to *Pax6* with *Nk2.2* in the spinal cord) and overlapping with *Otx* (Boncinelli et al., 1993; Simeone et al., 1993; Simeone et al., 2002), ventral *Six3* (Lagutin et al., 2003; Oliver et al., 1995) and dorsal *Emx* (Boncinelli et al., 1993; Simeone et al., 1992) and *Ngn* which promotes neuronal specification (Nieto et al., 2001) – a pattern which is to variable extent conserved across vertebrates, hemichordates (Lowe et al., 2003), arthropods (Lichtneckert and Reichert, 2005) and annelids (Steinmetz et al., 2010; Tomer et al., 2010). Wnt signalling in telencephalon positively regulates dorsal *Ngn* (and thus promotes neurogenesis) and *Emx* and negatively regulates ventral *Nk2.1* (Backman et al., 2005). The anterior-most tip of neuroectoderm with the apical organ of planktonic larvae and its corresponding part of the CNS is typical by an inhibition of Wnt signalling by secreted Wnt antagonists (see the section 7.1.3.2) leading to a lack of Wnt signalling activity and is devoid even of the expression of *Six3*, a signature conserved from Cnidaria to Bilateria (Marlow et al., 2014; Sinigaglia et al., 2013).

7.2.3.4. Peripheral nervous system and conclusion

Unlike the central nervous system, the early specification of neurogenic domain in the peripheral nervous system is rather potentiated than inhibited by BMP signalling (Lu et al., 2012; Ota and Ito, 2006) and the Wnt/β-catenin signalling is active in sensory neuron precursors in both migrating neural crest (Lee et al., 2004) and non-migrating epidermal cells , similar to the situation in the dorsal nerve cord.

We can conclude that the Wnt/β-catenin signalling endows ectodermal cells with general neural characteristics. It is thus important for the early specification of the neuroectoderm and for the production of neural progenitors (neurogenesis) across the entire nervous system. Although its gradient is used for the specification of neuronal types, it is always achieved in a co-operation with another signalling pathway.

7.2.4. Digestive tract

Some kind of digestive system is present in all metazoan groups except Placozoa, that have an external digestion. A through gut with anterior oral and posterior anal openings was traditionally considered to be one of the diagnostic features of Bilateria. However, sponges possess a system of aequiferous canals that connect the gastric cavity to the external environment and ctenophores can even use sphincters to evacuate waste through their anal pores (Presnell et al., 2016). On the other hand, acoels (bilaterians) do not possess any anal opening so they have only mouth (Hejnol and Martindale, 2008). A gut is formed in the process of gastrulation in which endodermal and mesodermal precursor cells get from the surface inside the embryo. The gastrulation takes place at the vegetal pole and the gut is thus derived from the blastopore. Some animal phyla derive from blastopore the anus (a deuterostomous development), while the others the mouth (a protostomous development) or both (an amphistomous mode of development). The homology of digestive tracts and especially of their openings is hence complicated to assess and so far remains unresolved. The Wnt/β-catenin plays an important role in the embryonic development of the digestive tract from the very beginning, as well as in its function in the adulthood. Understanding of its involvement in these processes and comparative analysis of its functions in various animal phyla thus might be crucial for deciphering the gut evolution.

7.2.4.1. The specification of endoderm and the formation of a through gut

As was already described in the section 7.2.1.1, the activity of Wnt/β-catenin signalling on the vegetal pole of an egg early in development is responsible for the separation of mesendoderm (a common precursor that of endoderm and mesoderm) from ectoderm.

In vertebrates, the endoderm is formed by a regulatory cascade controlled by Nodal/Activin-related molecules from TGF-β family and leads to an activation of the gene *Sox17* (Alexander and Stainier, 1999; Xanthos et al., 2001), a master regulator of vertebrate endoderm (Hudson et al., 1997; Kanai-Azuma et al., 2002). However, Nodal expression is activated by Wnt/ β-catenin signalling early on (unless they are maternal like VegT or Vg1; see the preceding sections 7.1.5.2 and 7.2.3.1) which implies that Wnt signalling initiates this cascade. Wnt signalling can also directly promote expression of *Sox17* (see the chapter

7.1.5). Moreover, β-catenin associates directly with Sox17 and potentiates the expression of other downstream endoderm-specific genes, like *FoxA* (Sinner et al., 2004). Consequently, Activin A together with Wnt3A are the most successful cocktail for differentiation of definitive endoderm from embryonic stem cells (Toivonen et al., 2013). Wnt/β-catenin signalling and *Sox17* expression is inhibited only in the anterior (visceral) endoderm to establish the A-P axis polarity (Perea-Gomez et al., 2001; Zorn et al., 1999b). FoxA is also called HNF3 and belongs to the Fox/forkhead family of winged-helix transcription factors (TFs) that unites Forkhead and mammalian hepatocyte nuclear factors (HNF) with FOXO (Mazet et al., 2003) and. The expression of *HNF3* is characteristic of definitive (gut) endoderm (Ang et al., 1993; Monaghan et al., 1993), whereas HNF4, GATA4 and GATA6 are required for the formation of anterior visceral endoderm (Morrisey et al., 1998; Soudais et al., 1995). GATA TFs then co-operates with Nk2 proteins in the regulation of target genes (Durocher et al., 1997; Zhang et al., 2007). *HNF3* and *Nk2.1* are expressed in the developing gut also in amphioxus (Shimeld, 1997; Venkatesh et al., 1999). In urochordates, Wnt signalling promotes the expression of endoderm specific genes and represses ectodermal regulatory transcription factor, surprisingly GATA4/5/6, which in other animal phyla promotes endoderm formation (Rothbacher et al., 2007). β-catenin triggers the formation of endoderm by a direct positive transcriptional regulation of *FoxA* (Hudson et al., 2016) and *Lhx3* homeobox and through them indirectly of an *Otx* homologue and an *Nk*-class gene (Oda-Ishii et al., 2005; Satou et al., 2001) in the ascidian *Ciona savignyi*, although the latter two are dispensable for endoderm formation despite *Nk2* is able to induce expression of endoderm markers and convert notochord to endodermal fate (Ristoratore et al., 1999; Spagnuolo and Di Lauro, 2002). The gut in chordates is formed by an invagination followed by involution of epithelium (in anamniotes) and/or the ingression of the cells through the primitive streak (in amniotes) (Solnica-Krezel, 2005; Stower and Bertocchini, 2017). According to the traditional view, the blastopore gives rise to the anus and the mouth breaks open secondarily.

In Echinodermata, maternal Wnt signals trigger the regulatory circuit of Wnt8 signalling that encompasses the transcription factors *Blimp1*, *Krox* and *Otx* (Smith et al., 2007; Yuh et al., 2004) and induction by Activin (Sethi et al., 2009). The Wnt and Activin signalling upregulate the expression of transcription factors *Brachyury*, *GataE*, *FoxA* and *FoxB*, among others (Davidson et al., 2002). The gut arises by an invagination, the blastopore gives rise to the anus and the mouth is formed secondarily (Martik and McClay, 2017).

In *Drosophila*, Wingless signalling regulates the expression of homeobox transcription factor *defective proventriculus* which is required for endoderm development (Fuß and Hoch, 1998; Nakagoshi et al., 1998) and lacks any vertebrate homologue. Further endodermal differentiation is dependent on GATA transcription factors serpent (Reuter, 1994) and dGATAe (Okumura et al., 2005) and forkhead/HNF3 (Weigel et al., 1989a). *GATA* genes are activated by a terminal Torso (Tor) signalling activity (Murakami et al., 2005). mTOR (a mouse Tor homologue) signalling can be activated as an alternative branch of canonical Wnt signalling by the inhibition of GSK-3β (Inoki et al., 2006), raising the possibility that also *GATA* expression is potentiated by Wnt. The gut arises from two (anterior and posterior) endodermal primordia separated by ventral mesoderm which is internalized in the ventral furrow. The endodermal primordia at the anterior and posterior termini of the ventral furrow infold and the oral and anal invaginations fuse to form the gut tube (Leptin, 1999). The genes *caudal*, *forkhead* and *wingless* seem to constitute a core module for the formation of a hindgut invagination. Furthermore, *forkhead* and *wingless* are present also in the oral invagination (Wu and Lengyel, 1998).

HNF3 and HNF4 Forkhead TFs and GATA4/5/6 are involved in the gut formation also during the amphistomous gastrulation of Onychophora (Janssen and Budd, 2017), more basally branching arthropods, in which the two blastoporal lips by epiboly envelope the endoderm from two sides and fuse at the midline, leaving the frontal and caudal openings for both the mouth and the anus.

The segregation of endoderm progenitor E from the common endomesodermal precursor EMS in nematodes and its regulation by Wnt signalling has been already described in the section 7.2.1.1, as well as the specification of endoderm in molluscs and nemerteans. In *Caenorhabditis elegans*, POP-1/Tcf activated by Wnt signalling in the E blastomere co-activates the expression of downstream *end-1* and *end-3* GATA proteins (Maduro et al., 2005) and by their means ELT-2, an Nk2 type homeodomain TF that in turn regulates *pha-4*, an HNF3β/forkhead transcription factor (Horner et al., 1998; Kalb et al., 1998).

Annelids express *Brachyury*, *FoxA*, *GATA4/5/6*, *Blimp1*, *Nk2.1* and *Otx* in the endodermal tissue of their blastopore and the developing gut (Boyle and Seaver, 2008; Boyle and Seaver, 2010; Boyle et al., 2014; Nardelli-Haefliger and Shankland, 1993). Vegetal macromeres are internalized by the epiboly of ectoderm and become part of the midgut (Meyer et al., 2010), while the blastoporal lips fuse in the ventral midline and give rise to both the mouth and the anus by the amphistomous mode of gastrulation.

To conclude, Wnt signalling is on the top of the signalling cascades that lead to endoderm formation in all phyla. The early segregation of endoderm lineage is regulated by the expression of transcription factors from Fox/Forkhead family transcription factor *FoxA* and GATA transcription factor(s) *GATA4/5/6* and the downstream endoderm specification programme often entails an activation of *Blimp1* and neurogenic genes *Otx* and *Nk2.1*. They are expressed initially in the vegetal blastomeres, the blastopore and continue to be expressed in the gut.

7.2.4.2. A-P patterning and compartmentalization of the digestive tube

The alimentary canal differentiatiates along its A-P axis to several functional compartments. Most animal groups possess a tripartite gut, which means that the gut becomes subdivided into three major distinctive parts: the foregut (or pharynx), the midgut and the hindgut. The midgut confers main digestive and enzymatic functions and for this purpose its endoderm differentiates to a secretory epithelium.

Some of the genes expressed ubiquitously during the specification of endoderm are later restricted to specific compartments along the gut's A-P axis and participate on their differentiation. *FoxA* and *Otx* can be always found in the foregut, e. g. in the annelid *Chaetopterus* and the sipunculan *Themiste* (Boyle and Seaver, 2010), but the overall pattern of expression is broader. As mentioned before, *Otx* is also involved in a regionalization of the anterior neuroectoderm (section 7.2.3.3). *FoxA* is expressed in both terminal parts of the gut, the foregut and the hindgut, in *Drosophila* (Weigel et al., 1989a; Weigel et al., 1989b) and the oligochaete annelid *Capitella* (Boyle and Seaver, 2008) and in both terminal parts of the gut plus the ventral midline (i. e. the blastoporally derived tissue in annelids) and later in the entire in polychaete annelids (Kostyuchenko et al., 2018). Either *FoxA* and *Otx* in annelids also display some segmental expression (Boyle et al., 2014; Kostyuchenko et al., 2018). *GATA4/5/6* is expressed in the midgut endoderm, as is the case in insects (Okumura et al., 2005; Reuter, 1994) and some annelids (Boyle and Seaver, 2010). In organisms with more *GATA* genes, as the annelid *Capitella*, different *GATA* genes can specify certain gut compartments (Boyle and Seaver, 2008). *Nk2.1* was observed in the foregut, hindgut and isolated cells of the midgut endoderm in the annelid *Capitella teleta* (Boyle et al., 2014), whereas it is activated in a relatively small ventral-medial area in the endodermal tube in vertebrates and regulates the lung and thyroid development (Pera and Kessel, 1998). Nevertheless, the remaining *Nk2* paralogues are involved in the development

of endoderm-derived organs in foregut and midgut regions of vertebrate gut tube – the pharynx (Tanaka et al., 2001), the small intestine (Pabst et al., 1999) and pancreatic β-cells (Sussel et al., 1998) – and their combined expression pattern hence quite closely corresponds to that of *Nk2.1* in the annelid.

Alimentary canals of many organisms (e. g. vertebrates) display a higher degree of specialization and demand finer regionalization than what can be provided by a simple tripartite gut. The boundaries between gut compartments might be established by interactions among compartment-specific transcription factors and/or in a response to A-P gradients and display elevated activity of the canonical Wnt/β signalling in *Drosophila* (Buchon et al., 2013). Every germ layer, including endoderm, is patterned along its A-P axis by the expression of homeobox genes (Beck, 2002). An enteric Hox code has been identified (Kawazoe et al., 2002; Pitera et al., 1999) which acts primarily on the visceral mesoderm of the gut wall that in turn differentiates endoderm. However, *ParaHox* genes, especially *Cdx/caudal*, seem to be key regulators of the gut development and A-P patterning (Beck and Stringer, 2010) as they are direct transcriptional targets of Wnt/β-catenin signalling (Lickert et al., 2000; Pilon et al., 2006; Pilon et al., 2007) and act upstream of *Hox* (Beck et al., 2000; Davidson et al., 2003; Charité et al., 1998) to mediate to them the positional information of the A-P Wnt gradient from the Wnt proteins emitted by a posteriorly located Wnt signalling centre (e. g. Wnt3A).

A compartmentalization of gut by transcription factors leads to a differential activation of signalling pathways and expression of effector genes which results in the differentiation of compartment-specific cell types. This includes also a localized activation of Wnt/β-catenin signalling and a compartment-specific expression of canonical Wnt components. For example, the canonical Wnt signalling shows elevated activity and *Frizzled3* expression on the compartmental boundaries in the adult *Drosophila* gut (Buchon et al., 2013) but it is also present to a lower level in the midgut epithelium (Tian et al., 2016). Most canonical Wnt signalling components, including two *Tcf* paralogues *Lef1* and *Tcf4*, are initially expressed in the visceral mesoderm of foregut and hindgut compartments of the developing chicken gut (Theodosiou and Tabin, 2003). Also a single amphioxus *Tcf* gene is expressed in the pharynx (foregut) and hindgut during development (Lin et al., 2006). On the other hand, mammalian *Tcf4* is expressed in the small intestine (i. e. the midgut) during development, while *Tcf3* transcripts can be found in the stomach epithelium (a foregut derivative) (Barker et al., 1999). In chicken, some components of Wnt/β-catenin/Tcf signalling progressively become expressed also in the endoderm of either the

small and the large intestine where they in mammalian gut in fine patterns (Gregorieff et al., 2005) promote the proliferation of enterocyte progenitors and a renewal of the digestive epithelium (see the following section). It thus seems, that the differences in expression of Wnt components can be attributed to the examined developmental stages as well as to species differences and that there is significant spatiotemporal dynamics in the expression of Wnt pathway components and the Wnt/β-catenin signalling activity during the intestinal development. In conclusion, an initial Wnt expression and activity lies in the foregut and the hindgut, while it later expands to the midgut region.

7.2.4.3. Differentiation and maintenance of the digestive epithelium

The epithelial lining of an alimentary canal is exposed to a harsh chemical and mechanical stress imposed on it by ingested food, toxins, secreted digestive enzymes, detergents and extreme pH. Although the digestive cells are well adapted and protected, they still have only very short life span and have to be replenished by proliferation and differentiation of progenitors generated in stem cell niches interspersed throughout the epithelium. The process of cell differentiation in the gut thus does not stop after the embryonic development has been terminated but instead continues even in the adulthood. The best known is the renewal of epithelium in the adult mammalian small and large intestine. However, it is probable, that there are only subtle differences when compared to the differentiation in the embryonic development. Since Wnt signalling plays a major role in the intestinal epithelium maintenance and its deregulation often leads to cancer, it was also the most studied and contributed a great deal to our knowledge of canonical Wnt/β-catenin signalling pathway.

In the mammalian intestine, new epithelial cells are generated in Wnt positive stem cell niches that are located at the bottom of the intestinal crypts (Gehart and Clevers, 2019). Wnt3, Wnt11 and EGF signal produced by Paneth cells (Sato et al., 2010) and BMP inhibitors, Wnt and R-spondin signals secreted by the underlying mesenchyme (Aoki et al., 2016; Stzepourginski et al., 2017; Valenta et al., 2016) are received by neighbouring cells, maintaining them as the intestinal stem cells (ISCs). They can be identified by their expression of *Lgr5*, a well-known intestinal stem cell marker (Barker et al., 2007) and a co-receptor for R-spondin, a potent enhancer of Wnt/β-catenin signalling (see the section 7.1.4.2). The ISCs divide symmetrically but due to a limited space in the stem cell niche compete for available signals and some daughter cells retain their stemness, the others give

rise to fast dividing enterocyte progenitors (Lopez-Garcia et al., 2010; Snippert et al., 2010). By continued cell divisions, new cells are moved towards the crypt opening, where the Indian hedgehog signalling from the mature colonocytes (van den Brink et al., 2004; van Dop et al., 2009) and the BMP signals from the colonocytes (Hardwick et al., 2004) and the mesenchyme (Haramis et al., 2004) oppose Wnt signalling and cause the differentiation of gut progenitor cells; however, their fate is determined already in the stem cell niche by activation of Notch signalling (see further in the text below). Therefore, Wnt/β-catenin signalling activity is highest at the bottom of the crypts and lowest at the tips of villi/between the crypts and enterocytes differentiate along its gradient.

Both *wingless* and *hedgehog* are expressed on the boundaries between major gut compartments in insects (Buchon et al., 2013; Hoch and Pankratz, 1996; Inoue et al., 2002). The maintenance of gastric stem cells requires Wnt signalling activity (Strand and Micchelli, 2011) and a hyperactivation of the Wnt/β-catenin signalling by inactivation of *Apc* genes in *Drosophila* causes an epithelial hyperplasia due to an over-proliferation of intestinal stem cells (Lee et al., 2009). The *Apc* mutations may lead to a formation of tumours in the fly's gut just as they do in vertebrates (Martorell et al., 2014; Wang et al., 2013) but their effect can be reverted by a disruption of the Wg signalling (Lee et al., 2009; Wang et al., 2013). The stem cells are maintained by visceral muscles which produce Wg that is necessary for ISC self-renewal (Lin et al., 2008), EGF that drives cell proliferation during development (Jiang and Edgar, 2009) and adulthood (Jiang et al., 2011) and BMP which limits the proliferation (Guo et al., 2013). Also Hedgehog is expressed in the gut epithelium in the late developmental stages of the leech *Helobdella robusta* (Kang et al., 2003). Together, all these observations suggest that the mechanism of the maintenance of digestive epithelium by a production of undifferentiated progenitors regulated by Wnt/β-catenin signalling and their subsequent differentiation to the absorptive cells might be evolutionarily ancient and conserved across animal phyla (Takashima et al., 2013). Conversely, according to more recent findings, a low level Wnt signalling is active in enterocytes throughout the gut epithelium in *Drosophila* and prevents JAK-STAT signalling in neighbouring ISCs from promoting the proliferation (Cordero et al., 2012; Jiang et al., 2009; Tian et al., 2016). There are indications that a similar mechanism operates also in mammals (Cordero et al., 2012) and so it more likely represents a break against overproliferation, even more necessary in insects because Hh engages in a positive (rather as negative as in vertebrates) feedback with Wg signalling, as demonstrated by their mutual activation on the parasegmental boundary (cf. the section 7.2.2.2).

Despite being the most numerous, absorptive cells are not the only cell type in the gut. Specialized cell types produce digestive enzymes, mucous and acidify the lumen of gastric part of the digestive tract (exocrine cells), cells that produce hormones (enteroendocrine cells). Secretory cell types express Notch ligands (upregulated by Wnt signalling, see the section 7.1.5.2) that activate Notch pathway in the surrounding cells, force them to override the default secretory fate and become enterocytes (Crosnier et al., 2005; van Es et al., 2005). Exocrine and enteroendocrine cells express neurogenic and pro-neural genes during differentiation (Gehart et al., 2019) and individual cell types differ by their expression of different Delta, Delta-like and Jagged ligands for Notch (Zecchin et al., 2007). The same system is functioning also in the specification of the acid-secreting copper cells in the gastric region of the *Drosophila* midgut – Wg signalling is active in copper cells and Notch in surrounding interstitial cells (Tanaka et al., 2007). These distinct cell types probably have emerged in the evolution by a division of labour from a common ancestral cell type and all these functions might be in simpler digestive systems still provided by a single cell type. Nevertheless, it seems that the main role of Wnt/β-catenin signalling in the gut lies in the maintenance of ISCs and the cell fate decisions and differentiations are results of interactions with other signalling pathways.

7.3. Model organism *Platynereis dumerilii*

7.3.1. General description

Platynereis dumerilii (Audoin & Milne-Edwards, 1833) is a marine polychaete annelid [phylum Annelida, class/clade Errantia, order Phyllodocida, family Nereididae; the former class Polychaeta was polyphyletic (Struck et al., 2011)], a representative of a spiralian/lophotrochozoan lineage of protostomous bilaterian animals [Metazoa: Bilateria: Protostomia: Spiralia/Lophotrochozoa (Dunn et al., 2014; Telford et al., 2015)]. Because the polychaete annelids have relatively few phylum-specific synapomorphies and their morphology has not changed significantly since the Cambrian period, *Platynereis* proved to be a useful emerging animal model for the comparative studies of development and evolution (evo-devo) to reveal the evolutionary origins of bilaterian traits (Williams and Jékely, 2016).

The worms are omnivorous benthic feeders that live in silk tubes built on a substrate in shallow coastal waters on the sea shores around the globe, from the tropical to cold temperate climate including Europe (GBIF.org). They are indirect developers with highly stereotypic early development and an indeterminate growth with the adult worms reaching from 20 to 40 mm in length. They are gonochorists with marked sexual dimorphism, females being usually bigger and after reaching sexual maturity differently coloured than males. The external habitus is captured in the Figure 2. The diploid (2n = 28) (Jha et al., 1995) genome of about 1 Gbp with vertebrate-type intron-rich genes (Raible et al., 2005) has not yet been published, but transcriptomic database have become available in the course of this work (Chou et al., 2018).

7.3.2. Life cycle

The life cycle and morphology of *P. dumerilii* developmental stages have been described to a greater detail before (Fischer and Dorresteijn, 2004; Fischer et al., 2010). Hereafter, I extract and summarize key features and transitions in *Platynereis* life cycle (Figure 2) which are necessary for an understanding of the following text.

The speed of development is temperature-dependent (Fischer et al., 2010) but highly synchronous from the beginning. Therefore, the same stage is always reached by all larvae of the same batch and across different batches in the same temperature at certain time point.

The developmental staging has been done at the temperature of 18 °C, which became the standard cultivation experimental temperature of sea water for *Platynereis* and allows the use of age in hours or days post-fertilization to assign developmental stages. The larvae of *Platynereis* are lecitotrophic, do not feed and live the entire first week of their development from yolk provisions. The development is synchronous only until the start of feeding and growth, when it becomes nutrition-dependent and progressively more and more asynchronous.

The embryo develops inside a jelly coat during the first day after fertilization to a spherical planktonic trochophore larva that hatches around 24 hpf (hours post-fertilization). They are positively phototactic and use small larval ocelli and ciliary beating of a single circumferential ciliary belt to swim in a right-handed (clockwise; when viewed from the back) helix to regulate their movement in the water column (Jékely et al., 2008). During the metatrochophore stage (from 48 until 72 hpf), the spherical trochophore changes its shape to conical and then to torpedo-like, nearly simultaneously forms three larval segments (plus one cryptic fused to the episphere to form the head) and starts to use multiple new segmental ciliary belts for swimming. In the following nectochaete stage (from 72 hpf until the settlement) the larva further elongates, develops adult eyes, the trunk musculature and consequently a mobility of body appendages with chaetae, which help to avoid predation in a startle response (Bezares-Calderón et al., 2018) while the larva is still swimming by cilia. The larva becomes negatively phototactic, at first alternates between swimming and crawling between 5 to 7 dpf (days post-fertilization) and eventually definitively settles to the bottom in a process which is controlled by a myoinhibitory peptide (MIP) signalling among the cells of the apical organ (Conzelmann et al., 2013).

The settled larva crawls on the bottom and starts to feed the day 7 or 8 of development (Fischer and Dorresteijn, 2004) on benthic algae or flagellates. It adds new segments continuously and becomes an errant juvenile. Then, around 1 mpf (month post-fertilization) the first trunk segment fuses with the head during the cephalic metamorphosis. The atokous worm on a substrate builds a silk tube from a product of its spinal glands and includes larger food particles, which it collects from the surroundings, to its diet.

Dependent on the amount and quality of available food, the worms reach around 70 segments after 3 months to 1,5 year of life, stop feeding, commence a sexual metamorphosis and establish a sexual dimorphism. Their eyes enlarge and the appendages become paddle-like. Females convert most of their tissues to eggs and gain yellow colour from the egg yolk visible through a transparent thin body wall. One third of the male body anterior to the

gonopore becomes white and the posterior two thirds turn red. Sexually mature worms leave the tubes and engage in the mass swarming and spawning. *Platynereis* possess circalunar and circadian clock and most animals mature in the week after a full moon with a peak locomotor activity in night hours (Zantke et al., 2013). The worms during spawning swim speedily in circles, until the eggs are released through pores made by ruptures in the body wall into the sea water. The fertilization is external and one female can produce up to 3000 eggs (Fischer and Dorresteijn, 2004).

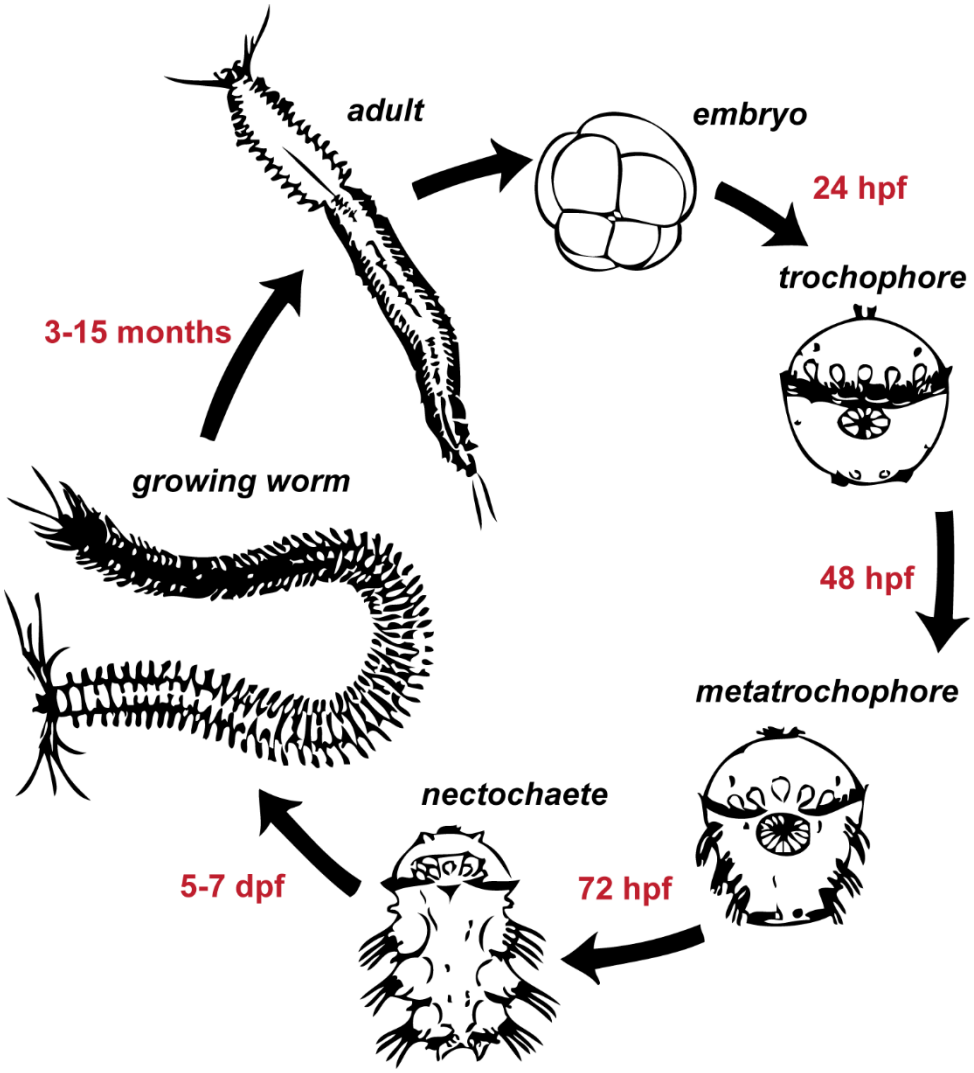


Figure 2 – Life cycle of *Platynereis dumerilii* (Audoin & Milne-Edwards, 1833)

Adapted from (Saudemont et al., 2008), description in the text. Depicted larvae correspond to intermediate stages between the time points indicated near arrows (*red*) which were used in this work.

7.3.3. Larval morphology and anatomy

The descriptions of larval adult stages are based on those published before (Fischer et al., 2010) and own observations.

7.3.3.1. Trochophore

The approximate living trochophore larva of *P. dumerilii* (between 24 hpf and 48 hpf on the Figure 2) is ball-shaped with the diameter around 150 μm (it slightly shrinks after the fixation and staining procedures). It is divided by prototroch, an equatorial circumferential ciliary belt from 12 multiciliated cells, to two halves of approximately the same size, the anterior episphere and the posterior hyposphere. The episphere corresponds to the future frontal part of the head (the acron or prostomium) and contains cerebral ganglia and sensory organ. The anterior-most cells bear an apical tuft of sensory cilia, contain statocysts and together form a sensory apical organ (Marlow et al., 2014). Two small larval eyespots or ocelli of only a single photoreceptor and a single pigment cell are located on both lateral sides of the episphere. The photoreceptor cells have synapses directly on multiciliated cells of the prototroch and adjust the ciliary beating and hence the direction of movement based on illumination (Jékely et al., 2008). The axonal plexus of cerebral ganglia is not yet present in the early trochophore (24 hpf, not depicted on a scheme) but differentiates during the trochophore stage and can be found in the episphere by the end of the trochophore stage (48 hpf on the Figure 3, right).The trochophore's hyposphere (the future trunk) consists of four large yolk-rich cells enveloped by epidermal and neural ectoderm (the neuroectoderm). Each of the yolk cells contains a large lipid droplet. Stomodaeum, the future oral opening of the digestive tract, is located centrally on the ventral side just posterior to the prototroch and a ring of cells of the stomodeal rosette begins to form around (Figure 2; not depicted on the Figure 3). Head kidneys are located on both sides of the stomodaeum (Hasse et al., 2010). Almost no nerves can be observed in the early trochophore (24 hpf) but they appear during the trochophore stage as the neurons differentiate. Two axonal bundles of the ventral nerve cords (VNCs) run on the ventral side longitudinally along the ventral midline and converge in the posterior. By the end of the trochophore stage (48 hpf) there are 3 distinct transversal commissures between the VNCs. The posterior-most terminal part of the body is the pygidium, which is separated from the hyposphere by short ciliary belts, the telotrochs.

7.3.3.2. Metatrochophore

The metatrochophore larva (Figure 2; early metatrochophore 48 hpf, late metatrochophore 72 hpf stage on the Figure 3) is of conical shape. It retains all the morphological characters which are present in the trochophore (the apical organ with the apical tuft, the larval eyes, the prototroch and the telotroch, the ventral nerve cords and the brain ganglia) but already possesses segmental structures and additional ciliary belts connected to the differentiation and formation of larval body segments. Three pairs of ectodermal chaetal sacs develop in the hyposphere and start to produce chitinous chaetae (setae, bristles). These are hidden in the body in the early trochophore but protrude from the body wall in later metatrochophore stages. Three morphological larval segments are formed and each bears a bilaterally symmetrical pair of short ciliary bands, the paratrochs. There is also a cryptic "zero" segment without bristles or parapodia, which is fused to the future head (Steinmetz et al., 2011). Segments I to III are thus called chaetigerous or trunk segments. Between the zero segment and the prototroch around stomodaeum is located the peristomium field, which is from the trunk also separated by another pair of ciliary bands called metatrochs; in feeding larvae of other marine invertebrates they form a narrow feeding groove, an opposed-belt system for prey capture, but persist also in some non-feeding larvae (Pernet, 2003).

Adult eyes are can be found on the episphere but they are small and not yet fully developed. They are separated from the apical organ by ciliated cells of the akrotroch. The ventral nerve cords are interconnected by numerous commissures. In the front, they send circumoesophageal connectives to the cerebral neuropil and together close a circumoesophageal neural loop (Starunov et al., 2017), while in the posterior the VNCs are connected by an anal neural loop. The stomodeal rosette is fully formed and becomes slightly elongated with a slit-like oral opening. The proctodeal invagination appears at the posterior terminus.

7.3.3.3. Nectochaete

The nectochaete (Figure 2, 7 dpf on the Figure 3) has a slender, elongated body. The appendages (parapodia) on both sides of each chaetigerous segment are prominent and due to a developing body musculature either the parapodia and the trunk segments become

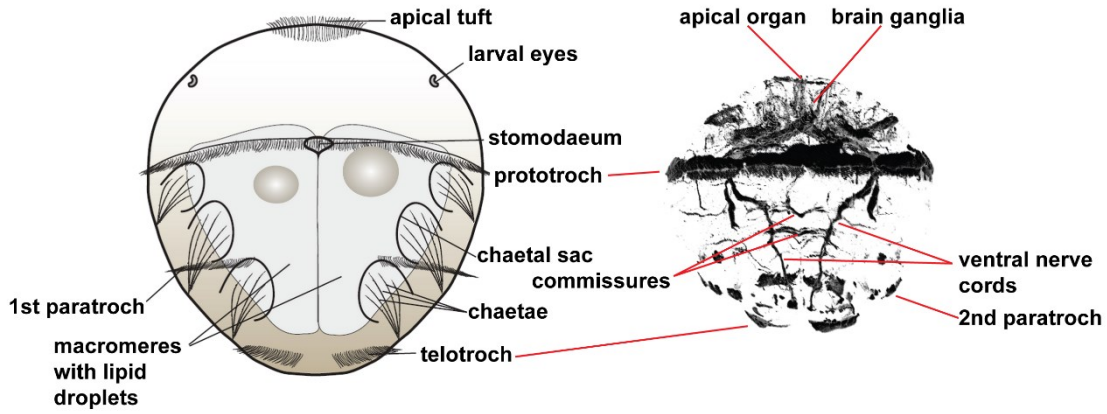
mobile. Paired ventral longitudinal muscles shorten the body and bear attachment of parapodial muscles. ventral oblique muscles attach to a single ventromedial longitudinal or axochordal muscle (the axochord) that runs just beneath the ventral midline (Brunet et al., 2016). This muscle has been by the expression of molecular markers *Brachyury*, *FoxA*, *Hedgehog*, *Noggin* and others homologized to the notochord of chordates (Brunet et al., 2015; Lauri et al., 2014). Primordial germ cells migrate from the posterior to the primary gonad on the dorsal side of the first chaetigerous segment (Rebscher et al., 2007).

The stomodaeum is transformed into an elongated muscular pharynx equipped with a slit-like oral opening and two chitinous jaws. The macromeres inside the trunk have cellularized (underwent multiple cell divisions) to the gut endoderm. Despite their remnants with lipid droplets still might be apparent, they created a lumen that is connected by communications to the pharynx and the posterior hindgut. The larva by the end of the nectochaete stage hence possesses a tripartite through alimentary canal which consists from an anterior foregut (pharynx), a central midgut and a posterior hindgut. Larval nephridia run parallel to gut on both of its sides between the parapodia of the second and third segment (Hasse et al., 2010).

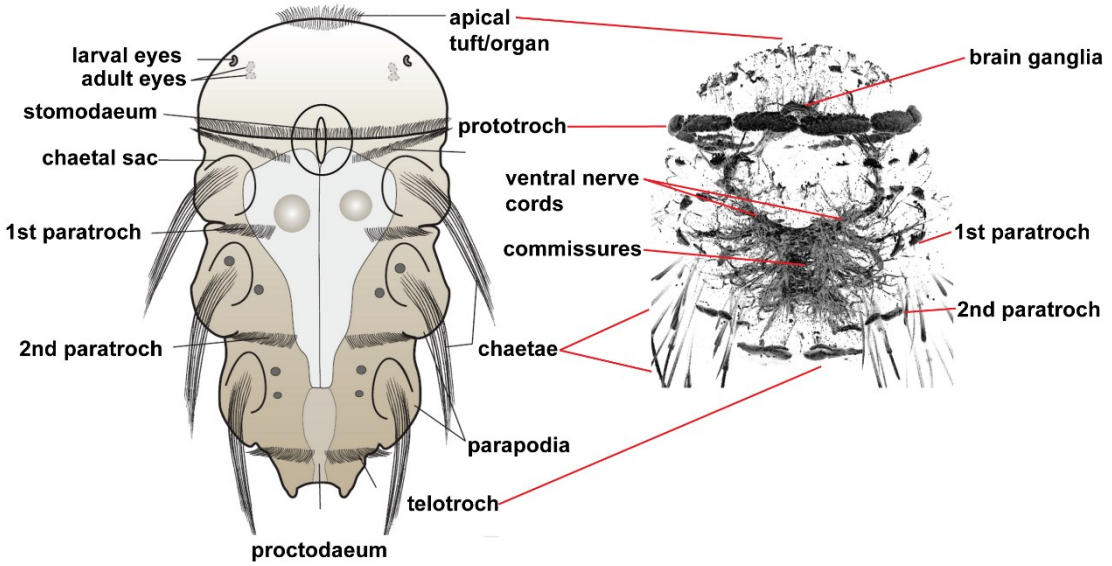
With the change of the lifestyle from swimming-planktonic to crawling-benthic, the prototroch degrades to two small ciliary bands on both sides of the head. Doublets of adult pigment-cup eyes on the dorsal side of the head are fully developed and functional while the larval eyespots are still present. The apical tuft is not apparent any more, but a pair of short antennae develops in the front of the head, a pair of flat palpae on the ventral side of the head and a pair of long anterior dorsal cirri. These organs bear tactile and chemosensory functions (Chartier et al., 2018). The pygidium bears a pair of similar protrusions, the aciculi.

Either the brain ganglia and the ventral nerve cords become more compact. Ventral nerve cords thicken and approach each other, being separated only by a narrow gap bridged by multiple commissures. The brain ganglia contain two pronounced dorsal mushroom bodies (Tomer et al., 2010) and extend nerves to the sensory organs, whereas the VNCs develop a suboesophageal ganglion and segmental ganglia that send segmental nerves to the parapodia (Starunov et al., 2017).

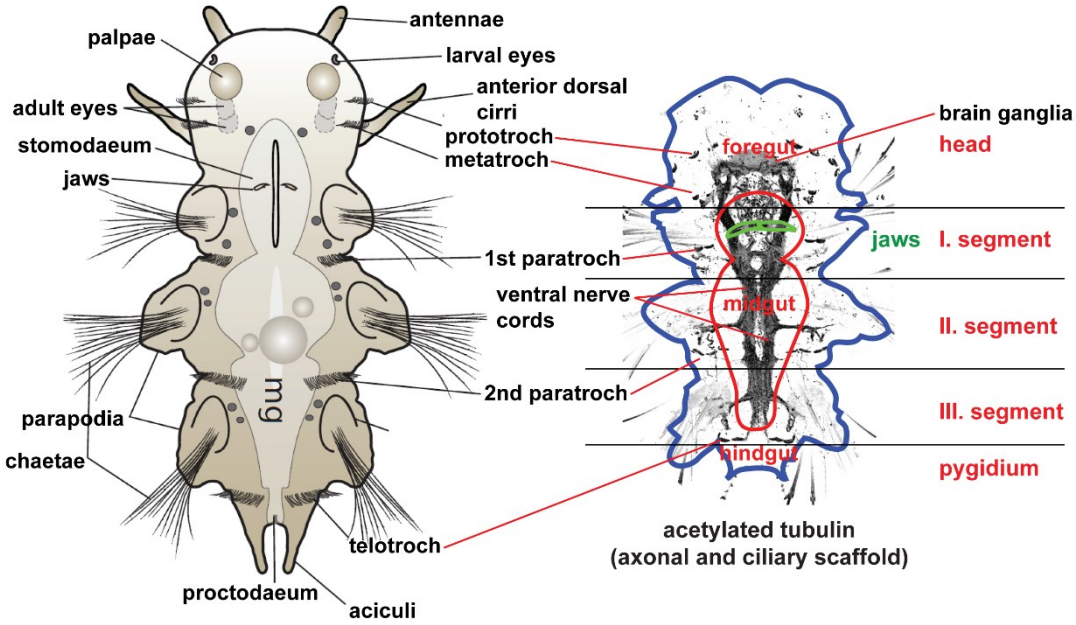
48 hpf



72 hpf



7 dpf



(on the previous page)

Figure 3 – Larval anatomy of *Platynereis dumerilii*

The anatomy of major developmental stages of *Platynereis dumerilii* life cycle used in this work.
48 hpf = a transition from late trochophore to early metatrochophore, 72 hpf = early nectochaete, 7 dpf = transition from late nectochaete to errant juvenile.
Prominent key morphological or anatomical features are indicated and some of them also related to the same structures on negative maximum projections of confocal *z*-stacks with acetylated α-tubulin immunofluorescent staining which labels stabilized form of tubulin in axons and cilia (depicted on the right). The stabilized form of tubulin is present in and thus visualizes the neuronal scaffold and ciliary belts of a larva. These axonal and ciliary scaffolds are used throughout the thesis as a reference for anatomical relationships of gene expression patterns or immunofluorescent stainings. For 7 dpf stage, outlines of body wall (*blue*), jaws (*green*) and digestive tract (*red*) are indicated, as well as the division of the body into 3 segments. Note that what is considered as a first segment is actually a second segment and the true first segment is incorporated into the head (Steinmetz et al., 2011); the same also happens later (around 1 mpf) during cephalic metamorphosis to the first chaetigerous segment.
Schemes on the left are modified from Fischer et al. (2010).

7.3.4. Juvenile to adult morphology and anatomy

The body has a variable number of segments which increases with age. Each segment carries paired definitive nephridia (Hasse et al., 2010) and one pair of parapodia and with its own innervation and musculature. The parapodium consists of a dorsal lobe (the notopodium) and ventral lobe (lobopodium) with multiple chaetae (hence Polychaeta). Cirri and antennae are long, the first chaetigerous segment has attached to the head during cephalic metamorphosis and carries a second pair of posterior cirri. The jaws are large and strong, with multiple dents. The ventral nerve cord, a pulsatile dorsal blood vessel (Fischer and Dorresteijn, 2004; Saudemont et al., 2008) and the gut run through the entire body up to the pygidium at the posterior terminus. The adults lack somatic gonads, although the germ cells are at first in gonial clusters behind the jaws (Rebscher et al., 2007) interconnected by cytoplasmic bridges and encapsulated in somatic sheath cells, but either are later lost and the germ cells float freely in the body cavity (Fischer and Dorresteijn, 2004).

7.3.5. Embryonic development

7.3.5.1. Embryonic cleavage

The first cleavage of the zygote at 18 °C occurs about two hours after fertilization. The early development of *Platynereis* is stereotypic, which means that cell fates are defined by their position in the cell lineage rather than by a position in the body. The fate and differentiation potential of cells are progressively narrowed down during development as they become determined autonomously by an unequally segregated maternal factors into individual blastomeres during embryonic cleavage. Therefore, cells can be traced by their developmental lineage to specific tissues and organs to which they contribute. They get to their positions in the body by a canonical fixed pattern of oriented cell divisions, which results in a fixed positions of signalling centres and stereotypic responses.

The egg is loaded with equally distributed components of asymmetrical cell division machinery which are polarized upon fertilization (Nakama et al., 2017). First two rounds of embryonic cleavage are perpendicular to each other and create four quadrants A-D. Third embryonic cleavage, perpendicular to the former two, creates two quartets of cells, vegetal yolk-rich macromeres and an animally located micromeres. The micromere quartet is twisted clockwise (when viewed from the animal pole) relative to the vegetal tier of macromeres. This tilt repeats in every following cell division, which results in a spiral pattern of blastomeres, hence spiral (or spiralian) cleavage. In *Platynereis*, nuclear β-catenin is observed in the vegetal blastomere of each sister cell pair during embryonic cleavage from the 8-cell stage until two transverse cell divisions establish the bilateral symmetry (Schneider and Bowerman, 2007). Precursors of future tissues thus acquire a unique pattern of activation, loss and reactivation of Wnt/β-catenin signalling. The third embryonic cleavage and the first with polarized nuclearization of β-catenin, segregates the β-catenin-negative animal tier of micromeres 1a-1d that constitutes the future anterior ectoderm of the episphere with the prototroch The episphere is completely ectodermal and originates from a characteristic group of cells called regarding its shape the annelid cross (Fischer and Dorresteijn, 2004). The vegetal tier of β-catenin-positive macromeres 1A to 1D gives rise to the posterior hyposphere (i. e. the trunk) and the head mesoderm. Next three rounds of cell divisions completely separate germ layers. The four quadrants of the vegetal tier are not equal and most of the tissues in the body are produced by the D quadrant, which establishes the D-V polarity. Ectodermal and neuroectodermal cells of the trunk are all descendants of 2a-2d micromeres, especially the somatoblast 2d. The endoderm

originates from the macromeres (4A-4D) (Ackermann et al., 2005) which represent the sole lineages that maintain a nuclear β-catenin throughout the entire development (Schneider and Bowerman, 2007). This highlights the importance of β-catenin and by extension of Wnt signalling for the development of endoderm and its derived tissues and organs. Yet, whether it is indeed necessary for the specification of endoderm was not demonstrated experimentally because only gain-of-function experiments with GSK-3β inhibitors have been done. The head mesoderm and mesodermal envelope of the stomodaeum are constituted by micromeres 3a-3d (Ackermann et al., 2005), while the bulk of trunk mesoderm and germ cells are descendants of the 4d micromere, the mesoblast (Fischer and Arendt, 2013; Özpolat et al., 2017). Interestingly, the polarity of β-catenin stabilization is reversed in the descendants of 4d mesoblast. The anterior β-catenin-positive cells in the 4d lineage form the mesoderm while their posterior β-catenin-negative sister cells become the primordial germ cells (Schneider and Bowerman, 2007).

7.3.5.2. Gastrulation

The embryonic cleavage gives rise to a compact stereoblastula. Descendants of the dorsally located 2d somatoblast start to migrate from both lateral sides as two ectodermal blastoporal lips and envelope by epiboly the entire hyposphere. The closing blastoporal lips result in a stereogastrula with a slit-like blastopore. The blastoporal lips eventually fuse in the ventral midline and leave only an anterior communication for the stomodaeum, while a posterior blastoporal tissue forms the anus. The mode of *Platynereis* gastrulation is hence amphistomous and both gut openings are derived from the blastopore (de Rosa et al., 2005).

The blastopores of *P. dumerilii* and of closely related polychaete *Alitta virens* express *Brachyury* (Arendt et al., 2001), *FoxA* (Kostyuchenko et al., 2018), *Even-skipped* and *Cdx*/*Caudal* (de Rosa et al., 2005), of which *FoxA* displays an affinity to the anterior part of the blastopore, while the other genes to the posterior. Consequently, they continue to be expressed in the respective blastopore-derive tissues: in the trochophore and metatrochophore stages, they are present in the ventral midline and the stomodaeum for *FoxA* or the posterior ectodermal cap (the pygidium) for the other genes. Later, in the nectochaete stage, these genes become to be expressed in the gut (see the section 7.3.7.3).

7.3.6. Wnt signalling in *Platynereis dumerilii*

The *Wnt* gene family of *Platynereis dumerilii* possesses 12 out of 13 described *Wnt* gene classes (Janssen et al., 2010; Prud'homme et al., 2002) that were present already in Cnidaria (Kusserow et al., 2005; Lee et al., 2006), except the canonical Wnt3 (which is lost in all protostomes) but unlike vertebrates they possess the WntA (Holstein, 2012). *Platynereis Wnt* genes display unique, but overlapping expression patterns (Pruitt et al., 2014). Their combined expression pattern encompasses the blastoporal lips in the hyposphere and two adjoining lateral fields in the episphere at about 16-24 hpf (Marlow et al., 2014; Pruitt et al., 2014). By the end of the trochophore stage/the beginning of the metatrochophore stage (at 48 hpf) when the blastoporal lips have already fused ventrally in the hyposphere, some *Wnt* genes are expressed in the stomodaeum, in the ventral midline and/or the pygidium, i. e. the descendants of the blastopore, and any of them are present non-identical segmental patterns. At the same time, *Wnt* genes that are present in the episphere with the exception of *Wnt4* preserve their expression in the lateral fields (Pruitt et al., 2014; Tomer et al., 2010). Unfortunately, nothing is known about the expression of *Wnt* genes in the later metatrochophore and nectochaete stages. In juvenile and growing worms, many *Wnt* genes are activated during the posterior growth and are expressed segmentally (Janssen et al., 2010; Prud'homme et al., 2003).

Wntless, which is a cargo receptor for Wnt proteins (section 7.1.2.2) and thus reflects the site of Wnt secretion, is expressed in ventral midline and segmental stripes at 55 hpf. On the other hand, *Pdu-Axin* is a gene whose protein product is a member of the β-catenin destruction complex (section 7.1.4.1), but also a positive transcriptional target of Wnt signalling (section 7.1.5.1) , hence it marks the area of Wnt pathway activation. The transcripts are detected in the proliferating cells along the ventral midline at 55 hpf and the expression is downregulated upon Wnt/β-catenin signalling inhibition (Demilly et al., 2013).

An activation and strength of a signalling pathway is given not only by a position of signal sources but also by a presence and amount of their transducers and effectors that have a permissive and modulatory role on the signal output. *Platynereis* possesses 4 genes for Frizzled receptors, three Frizzled-related proteins and two genes for sFRP. The genes for “canonical” Frizzled receptors are expressed broadly in most of the episphere and hyposphere. One of them *Pdu-Fz4* shows a more distinct pattern in the nectochaete stage (4 and 5 dpf), when it is can be found in the lateral episphere, around the oral opening and

on the foregut-midgut and midgut-hindgut boundaries (Bastin et al., 2015). Secreted Frizzled-related proteins are present in the apical organ (Marlow et al., 2014) and in the ventral midline (Bastin et al., 2015), probably to protect it from an autocrine signalling and activation of Wnt/β-catenin pathway.

The expression of the effector of Wnt signalling *Pdu-Tcf* used to be unknown until recently and is described as a part of this work.

7.3.7. Larval development and patterning

During the first week of development, the larvae do not accept food and therefore do not grow. Because of proceeding cell divisions while the larva retains its volume and only changes its shape, the cells are getting progressively smaller which enables morphogenesis and differentiation. Therefore, cell proliferation, morphogenesis, specification, patterning and differentiation of tissues and organs without growth occur during larval development of *Platynereis* until approximately 7 dpf at 18 °C.

7.3.7.1. Nervous system and sensory organs

The central nervous system of *Platynereis* develops in the episphere and on the ventral side of the trunk, where dorsal BMP signalling is most probably inhibited by Noggin produced by tissues which will later give rise to the ventromedial axochordal muscle, a putative homologue of the chordate notochord (Lauri et al., 2014). Therefore, the ventral ectoderm assumes a neuroectodermal fate. The fusion of blastoporal lips and hence a formation of the commissures between VNCs requires non-canonical Wnt/PCP pathway (Demilly et al., 2013). As the body of the larva changes its shape during development, the neuroectoderm narrows and elongates by a convergent extension via mediolateral cell intercalation, a process also directed by non-canonical Wnt signalling (Steinmetz et al., 2007). Non-canonical Wnt driven morphogenetic movements thus create a canonical Wnt signalling centre in the ventral midline.

Pdu-SoxB HMG-containing TF and *Pdu-NeuroD* bHLH transcription factor are expressed during neural progenitor commitment, while *Pdu-Neurogenin* (*Pdu-Ngn*) and *Pdu-Prox* are expressed broadly in the committed neurons and *Pdu-Collier* (*Pdu-Coe*), *Pdu-Ath* (*Atonal* homologue), *Pdu-Olig*, *Pdu-Achaete scute* (*Pdu-Ash*) bHLH TFs, *Pdu-SoxC* and *Pdu-Churchill* specify neuronal subtypes from nascent undifferentiated neurons (Demilly et al., 2011; Demilly et al., 2013; Kerner et al., 2009; Simionato et al., 2008). It was published in the course of this project that Pdu-Wnt4 produced by the ventral midline activate the Wnt/β-catenin signalling in the paramedial ectoderm, where it specifies *Pdu-Ngn*- and *Pdu-Ash*-positive neural progenitors of the CNS and stimulates their proliferation. The lateral PNS neural progenitors express in addition *Pdu-Olig* (Demilly et al., 2013). Unlike in other Bilateria, Notch nor its ligands are expressed in the neurogenic epithelium during the main stage of neurogenesis and play no role in general neurogenesis. Instead,

they are involved in the development of chaetal sacs and chaetogenesis (Gazave et al., 2017).

The patterning of the trunk neuroectoderm occurs between 24 and 48 hpf, when newly formed naïve neuronal progenitors are specified according to their position along a lateral to medial BMP gradient. Longitudinal neuronal progenitor populations are defined by their specific expression of *Pax* and *Nk* transcription factors in overlapping domains. From medial to lateral the neuroectoderm expresses in two paramedial domains *Pdu-Pax6*, then more laterally overlapping *Pdu-Pax3/7* and the most laterally lies the expression *Pdu-Pax2/5/8*. *Nk* genes are *Pdu-Nk2.2* is expressed along the ventral midline inside the *Pdu-Pax6* domains so that the longitudinal domains of *Pdu-Pax6* abut the medial *Pdu-Nk2.2*. Similarly, *Pdu-Pax3/7* expression, more lateral but overlapping with *Pdu-Pax6*, abuts medial *Pdu-Nk6* (Denes et al., 2007). The same pattern was described along the D-V axis of the vertebrate neural tube. Because the neuroectoderm of *Platynereis* does not fold into a neural tube, then (taking into account the D-V axis inversion in the chordate lineage) either the lateral-to-medial neural patterning system of *Platynereis* and the D-V neural patterning system of vertebrates seem to be homologues and place the origin of neuroectoderm with CNS-type patterning to the last common ancestor of all Bilateria (Arendt et al., 2008; Denes et al., 2007).

The A-P patterning of neuroectoderm is achieved by an anterior-to-posterior sequence of expression of homeobox genes. The apical plate of the episphere is characteristic by the expression of *Pdu-Six3* (Steinmetz et al., 2010). *Pdu-Otx* is expressed posterior to *Pdu-Six3* around the prototroch and stomodeum (Arendt et al., 2001; Steinmetz et al., 2011) and borders posteriorly with the expression of *Pdu-Gbx* in the cryptic segment (Steinmetz et al., 2011), similarly to the *Otx-Gbx* boundary between the vertebrate midbrain and hindbrain (see the section 7.2.3.3). The anterior boundaries of *Pdu-Hox1*, *Pdu-Hox4* and *Pdu-Lox5* are then located in the first, second and third larval segment, respectively (Steinmetz et al., 2011). The expression of *Pdu-Hox* genes in unaffected by manipulations of the retinoic acid signalling, which instead regulates neurogenesis and axon outgrowth in the medial neuroectoderm (Handberg-Thorsager et al., 2018).

Unlike in the trunk (the hyposphere), the neuroectoderm of the brain ganglia and sensory organs in the episphere contains to two lateral domains of Wnt expression which correspond topologically to the Wnt signalling centre in the dorsal telencephalon in vertebrates (Backman et al., 2005; Tomer et al., 2010). It has been shown, that the prominent cerebral ganglia of the annelid brain in the nectochaete stage, the mushroom

bodies, contain a gene expression fingerprint of *Pdu-Bf1*, *Pdu-Pax6*, *Pdu-Emx*, *Pdu-Dachshund*, *Pdu-Tailless*, *Pdu-Svp*, *Pdu-Achaete-scute* and *Pdu-Neurogenin* (*Pdu-Ngn*) which is similar to the one of the vertebrate cerebral pallium. Moreover, the lateral expression domains of *Pdu-Emx* expand in response to β-catenin stabilization by the inhibition of the GSK-3β by 1-Azakenpaullone (Tomer et al., 2010). However, in contrast to the situation in vertebrates (Machon et al., 2007), *Pdu-Pax6* expression in the expression seems not to be affected by ectopic activation of the Wnt/β-catenin pathway. The intermediate and medial parts of the brain (pars intercerebralis) express *Pdu-Gsx* and *Pdu-Nk2.1*, respectively, and the intermediate/medial expression domains of both these genes are reduced upon the pharmacological activation of Wnt/β-catenin signalling by 1-Azakenpaullone (Tomer et al., 2010) as they do in response to stabilization of β-catenin in mouse brain (Backman et al., 2005). Brain ganglia of *Platynereis* contain vasotocin-neurophysin-secreting and RFamidergic sensory-neurosecretory cells with a transcriptional signature of *Pdu-Pax6*, *Pdu-Nk2.1* and *Pdu-Rx* transcription factors identical to that of the cells that produce these hormones/neuropeptides in the hypothalamus in zebrafish (Tessmar-Raible et al., 2007). The apical organ develops in the anterior-most region which is devoid of *Pdu-Six3*, *Pdu-Otx* and *Pdu-Nk2.1* and expresses a combination of *Pdu-FoxJ*, *Pdu-Irx*, *Pdu-Nk3* and *Pdu-Hox1* (Marlow et al., 2014).

The developing eyes transitorily express *Pdu-Ath*, similar to the proneural sensory clusters. Larval ocelli differentiate in ventrolateral eye fields at the intersection of *Pax6* and *Six1/2* and the adult eyes' photoreceptors dorsally at the border of *Six1/2* in the close vicinity of cells permanently expressing *Pax6* (Arendt et al., 2002). *Platynereis* also harbours in its brain ciliary photoreceptors which are marked by the expression of *Pdu-Rx* and ciliary opsin (Arendt et al., 2004) and can override the positive phototaxis from the rhabdomeric eye photoreceptors to ensure an UV avoidance response (Verasztó et al., 2018).

7.3.7.2. Segmentation

First three segments in the larvae of *Platynereis* are formed almost simultaneously by a subdivision of the trunk and hence are called primary or larval segments. Consecutive secondary segments are added during post-larval development by an intensive proliferation and growth in the posterior growth/segment addition zone (PGZ, SAZ) between the last body segment and the pygidium. Even the pygidium itself exhibits some characteristics of

true segments, which was interpreted as that it is possibly of a metameric origin (Starunov et al., 2015). The SAZ expresses blastoporal marker genes *Pdu-Cdx*/*Caudal* and *Pdu-Eveness interrupted*, (de Rosa et al., 2005). The posterior growth zone contains stem cells with a transcription profile similar to primordial germ cells (Gazave et al., 2013), among others mesodermal stem cells, the mesoteloblasts (Fischer and Arendt, 2013; Özpolat et al., 2017). Mesoteloblasts are characterized by the expression of *Pdu-Vasa* and *Pdu-Nanos* and give rise also to the primordial germ cells that migrate to the anterior and colonize a primary gonad (Rebscher et al., 2012; Rebscher et al., 2007).

Based on segmental expression of *Pdu-Engrailed* and multiple *Pdu-Wnt* genes (Janssen et al., 2010; Prud'homme et al., 2003), it has been suggested that the segmentation mechanism is conserved between annelids and arthropods and that annelid segments correspond to arthropod parasegments. The larvae of *P. dumerilii* express putative canonical Wnt and an orthologue of *Wingless*, *Pdu-Wnt1*, but also *Pdu-Wnt11* in the posterior of each segment (Pruitt et al., 2014) where it abuts a thin stripes of *Pdu-Hh* and *Pdu-En* expression on the anterior of the following segments. Hedgehog receptor *Pdu-Patched* overlaps the segmental boundary while the TF that transmits the Hh signal, *Pdu-Gli* is expressed in a complementary segmental pattern inside the segments. The inhibition of Hh signalling by cyclopamine disrupts the segmental expression of these genes and downregulates either *Pdu-Hh*, *Pdu-Wnt1* and *Pdu-Wnt11* (Dray et al., 2010). This suggests that the same positive feedback loop between Wnt and Hh signalling exists on the segmental boundary as on the parasegmental boundary of insects (cf. the section 7.2.2.2); however, the function of Wnt/β-catenin signalling in segmentation has never been similarly experimentally verified. Interestingly, the NK genes *Pdu-Nk1*, *Pdu-Nk4*, *Pdu-Lbx*, *Pdu-Msx* and *Pdu-Tll* are expressed in segmental pattern as well but it is not clear if they have some role in segment formation and/or patterning (Saudemont et al., 2008).

The identity of larval segments in *P. dumerilii* and the closely related *A. virens* is determined by a *Hox* code, an A-P nested expression of *Hox* genes that, however, lack temporal collinearity (Kulakova et al., 2007). The integrity and exact order of the *Hox* cluster is not known but according to the numbering of *Hox* genes based on their closest homologues in taxa with intact *Hox* clusters indicates that the spatial collinearity is preserved. The A-P patterning of the trunk neuroectoderm was discussed in the previous chapter. Even before the onset of ectodermal expression, the anterior boundaries of *Hox2*, *Hox4* and *Lox5* specify the mesoderm of the first, second and third chaetigerous segments, respectively (Kulakova et al., 2017). All secondary segments added during the growth of

juvenile worms between the primary larval segments and the pygidium seem to carry the same *Hox* and *Wnt* expression (judged by the studies of tail regeneration, see further for citations). Nevertheless, there is a boundary between the anterior atokous (unchanged) and the posterior epitokous (metamorphosed) segments is revealed during sexual metamorphosis when it separates for example the white anterior third from the posterior red two thirds of the body of males. The position of this boundary is established even before metamorphosis. The underlying molecular mechanism remains unknown (Schulz et al., 1989) but it might coincide with the boundary between the expression of anterior (*Hox1*, *Hox4*) and posterior (*Hox7*, *Lox2*) *Hox* genes (Bakalenko et al., 2013).

Each segment develops paired parapodia. Each parapodium has a ventral and dorsal lobes called neuropodium and notopodium, respectively. Developing parapodia of a related polychaete *Neanthes arenaceodentata* lack the expression of genes *Distal-less*, *Dachshund* and *Optimotor blind* comparable to that seen in the forming appendages of arthropods (arthropodia) of vertebrates (Winchell et al., 2010) and *Distal-less* was not observed neither in the appendages in another annelid *Pomatoceros lamarckii* (McDougall et al., 2011). The appendages of *Platynereis* arise within parasegments (Dray et al., 2010) in contrast to their position on the boundary between parasegments in arthropods. Parapodia of annelids thus seem to be non-homologous to arthropodia or vertebrate limbs.

The genes expressed in the regenerative blastema and reconstituted SAZ after the amputation of the tail in adult nereidids include those present in the normal posterior SAZ like *Pdu-Cdx* and *Pdu-Eve* (de Rosa et al., 2005), multiple *Wnt* genes (Janssen et al., 2010) and *Hox* genes (Novikova et al., 2013; Pfeifer et al., 2012). The segment formation depends on a periodic expression of *Wnt1* alternating with *Hh* in the related nereidid *Perinereis nuntia* (Niwa et al., 2013). As a result, *Wnt*, *Tcf*, *Hh* and *Hox* genes are present in segmental pattern in regenerated segments with *Wnt* on the posterior of the segment and *Hh* on the anterior. This suggests that mechanisms of either normal segment formation and segment identity are from large part re-iterated during regeneration (de Rosa et al., 2005) and that Wnt signalling is involved in this process. Indeed, an artificially prolonged activity of Wnt/β-catenin signalling results in a widening of segments (Niwa et al., 2013).

7.3.7.3. Digestive system

As described above, the endodermal midgut of *Platynereis dumerilii* originates from a 4A-4D macromeres which are enclosed by epiboly of bastoporal lips inside the

hyposphere of a trochophore larva. These cells contain energetic provisions in the form of a yolk and lipid droplets. They eventually divide in the nectochaete stage and give rise to the midgut epithelium in the so called cellularization of the endoderm. The newly formed midgut forms a lumen and joins to the pharyngeal and proctodeal invaginations. New communications connect the midgut lumen with oral and anal openings and create a through tripartite gut, that consists of an anterior foregut (or pharynx), the digestive midgut and the hindgut (Fischer et al., 2010; Williams et al., 2015).

By a common transcriptional profile, the midgut cells can be traced back to just two cells that express *Hnf4* (Achim et al., 2018). The developing gut in the nectochaete stage of *P. dumerilii* and *Alitta virens* expresses also blastoporal marker genes. *FoxA* is expressed initially in the stomodaeum and the ventral midline, but progressively in both terminal parts of the gut, the foregut and the hindgut and eventually in all three parts of the gut (Kostyuchenko et al., 2018). *Pdu-Brachyury* is expressed in the stomodaeum/pharynx, several cells in the ventral midline and in the proctodaeum from 22 to 72 hpf, whereas *Pdu-Otx* expression is located to the peristomium and a part of the stomodaeum in the same stages (Arendt et al., 2001). In contrast, *Pdu-GATA4/5/6* seems to be active only in the mesoderm (Gillis et al., 2007).

The three parts of the digestive tube are functionally distinct and differ by an expression of effector proteins. At 6 dpf, few cells in the pharynx express *Pdu-Enteropeptidase*, a protease which activates other proteinases. The midgut at this stage expresses the genes *Pdu-Subtilisin-1* and *Pdu-Subtilisin-2* that encode peptidase, the gene *Pdu-α-Amylase* for the polysaccharide-digesting enzyme and the gene for the intracellular digestive enzyme *Pdu-Legumain protease precursor*, whereas the hindgut is positive only for *Pdu-Subtilisin-1* and *Pdu-Legumain* (Williams et al., 2015).

The developing gut displays an anterior-to-posterior overlapping collinear pattern of *ParaHox* genes *Pdu-Gsx*, *Pdu-Xlox* and *Pdu-Cdx* that could be potentially involved in its A-P patterning (Hui et al., 2009).

The gut wall gradually develops smooth muscles to ensure peristaltic movements and a passage of the ingested food. The ingestion of food and contraction of smooth muscles during peristalsis are controlled by an enteric nerve plexus with serotonergic neurons (Brunet et al., 2016) and the activity is controlled by the myoinhibitory peptide (MIP) secreted by sensory-secretory cells scattered throughout the entire length of the gut (Williams et al., 2015).

8 Research questions

In this thesis, I examine by pharmacological manipulations the role of Wnt/β-catenin signalling pathway in the larval development of the marine polychaete annelid *Platynereis dumerilii*, the representative of Spiralia.

My task is to explore the gaps and fill in some missing information in our current knowledge of the *Platynereis* Wnt/β-catenin signalling itself and its role in the development of three major distinctive features of bilaterian body plans:

A) central nervous system
B) body segmentation
C) digestive tract

The sequences and some expression patterns of *Platynereis* genes from the *Wnt* (Janssen et al., 2010; Pruitt et al., 2014) and *Frizzled* families (Bastin et al., 2015) and of *Pdu-Axin* (Demilly et al., 2013) have been published but the knowledge about other key components of the Wnt/β-catenin pathway is still missing. The Wnt effector transcription factor Tcf might determine, where the Wnt/β-catenin signalling is actually active and what is the outcome of the signalling.

The medio-lateral (M-L) patterning of the trunk neuroectoderm and its regulation by BMP signalling (Denes et al., 2007), as well as the A-P patterning of neuroectoderm (Steinmetz et al., 2011; Steinmetz et al., 2010) have been described for *Platynereis*. The expression patterns and the effect of BMP regulation were reported to be similar to those observed in the vertebrate and insect developing central nervous systems. However, it was not tested (except for the BMP regulation) how these patterns are generated in *Platynereis* and whether their regulation by Wnt/β-catenin signalling is also conserved. So far, the Wnt/β-catenin signalling was studied only in the *Platynereis* neurogenesis (Demilly et al., 2013) and in the regulation of few genes during the development of apical organ and mushroom bodies (Marlow et al., 2014; Tomer et al., 2010). Information about the Wnt regulation of neural patterning genes could potentially help to identify additional homologies between the *Platynereis* neuroectoderm and the vertebrate CNS, for example that of the isthmic organizer.

The segmentation mechanism of *Platynereis* has been proposed to be homologous to the one which operates on the parasegemental boundary of arthropods (section 7.2.2.2)

(Dray et al., 2010; Prud'homme et al., 2003). This proposed homology is based on the expression patterns and the functional experiment with Hh inhibition. Whether also the function of Wnt/β-catenin signalling on the segmental boundary is conserved was not tested, as well as the responsiveness of *Hox* genes to the Wnt/β-catenin signalling in the determination of the A-P segmental identity. The activity of Wnt/β-catenin signalling in the posterior segment addition zone is assumed in *Platynereis* based on the expression of *Wnt* genes in the pygidium (Janssen et al., 2010) but it was shown so far only in another polychaete *Perinereis nuntia* (Niwa et al., 2013).

Our only knowledge about the role of Wnt/β-catenin signalling in the *Platynereis* gut development is limited to the fate mapping studies which concluded that the endodermal digestive part of the gut, the midgut, develops from the macromeres of the fourth quartet (Ackermann et al., 2005) which are marked by nuclear β-catenin during every round of unequal cell division during the spiral cleavage (Schneider and Bowerman, 2007). Whether Wnt signalling has some further role in the endoderm specification is currently unknown.

Therefore, the questions which I aimed to answer were as follows:

1. Where is the Wnt/β-catenin signalling active during the *Platynereis* development?
2. How is this pattern formed and a how does it change during the development?
3. What are the properties and expression patterns of the *Platynereis Tcf* homologue?
4. Is Wnt/β-catenin signalling involved in the medio-lateral patterning of the neuroectoderm?
5. Is Wnt/β-catenin signalling involved in the antero-posterior patterning of the neuroectoderm?
6. Can be homologues of the vertebrate brain signalling centres identified in *Platynereis*?
7. What is the function of Wnt/β-catenin signalling on the segmental boundary?
8. Is the Wnt/β-catenin signalling active in the posterior segment addition zone?
9. Does the Wnt/β-catenin signalling pattern the gut along its A-P axis?
10. How is the Wnt/β-catenin signalling involved in differentiation and maintenance of the digestive epithelium?

9 Methods

9.1. Animal culture and spawnings

Worms in the desired larval and adult stages were collected from the wild type lineage of our established *Platynereis dumerilii* breeding facility at the Institute of Molecular Genetics of the Czech Academy of Sciences in Prague. Mature worms were mated in pairs in glass cylindrical containers filled with natural sea water (NSW). The mated worms were killed with EtOH and discarded several minutes after spawning and most of the volume in the containers was replaced with fresh NSW as soon as fertilized eggs settled to the bottom. The next day, I removed all poorly developing embryos from the bottom of the glass cylinder by two Pasteur pipettes and kept only the healthy population which used cilia to swim close to the surface of the water column. I kept the developing larvae designated for experiments in an incubator with a thermostat set at 18 °C to ensure the same staging as used by most of the authors (Fischer et al., 2010) while the adult worms were kept in the breeding room at 19 °C.

9.2. Transgenesis

9.2.1. Microinjections

Fertilized *P. dumerilii* eggs were washed thoroughly with 500 ml of filtered natural sea water (FNSW) and treated with 0.1 mg/ml proteinase K (Roche 03115828001) in FNSW for 25 seconds to permeabilize the eggshell. To stop permeabilization, the zygotes were rinsed quickly with half a litre of FNSW. Rinsed embryos were put in a 6-well plate and approximately 100 individuals transferred into a groove in a mould of 2% agarose/FNSW made in a 9.4 cm Petri dish by pouring the melted agarose under the form from two microscopic slides, the upper one with down-projecting ridge. The embryos were injected by glass capillaries (Eppendorf) while pressed against the higher left wall of the agarose groove and stripped off the capillary by incisions made in the right lower wall. The capillaries were pulled on a capillary puller with the following parameters: heat (h) = 465-490, pull (p) = 55-70, velocity (v) = 70, delay (d) = 180-200.

9.2.2. Wnt-responsive transgenic construct

For the original Wnt reporter construct, I first amplified SuperTOP-d1EGFP (a destabilized form of EGFP under Wnt responsive promoter containing 7 Tcf/LEF binding sites) from pd1EGFP-N1 with inserted SuperTOP promoter (courtesy of Ondřej Machoň) using primers with attached I-SceI restriction sites. The amplified product and pMos::r-opsin1::eGFP-F2A-NTR [courtesy of Vinoth Babu Veedin-Rajan (Veedin-Rajan et al., 2013)] were both digested with I-SceI, isolated on the agarose gel and ligated together. The resulting construct was confirmed by sequencing. Mos1 transpose mRNA was synthesized and purified by mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen™) from a KpnI-linearized pCS2-Mos template (courtesy of Vinoth Babu Veedin-Rajan). The concentration of the resulting Mos1 mRNA was then determined by Qubit™ RNA Assay Kit (ThermoFisher Scientific Q32852). The construct with the Mos1 transposase mRNA were injected each at 0,2 µg/µl concentration as a mixture together with 1:5 Fast Green FCF dye. However, I never saw a fluorescent signal with this construct nor I observed its presence by PCR after longer cultivation of injected worms (suggesting integration into the genome).

As a result, I decided to use simpler non-integrating transient Wnt reporter and injected a solution of 1:5 Fast Green FCF dye with 0.4 µg/µl SuperTOPFlash-tdTomato (courtesy of Vladimír Kořínek), which contains a gene for tdTomato fluorescent protein under the promoter with 8 Tcf/LEF binding sites. Thanks to these binding sites, this construct is responsive to Wnt/β-catenin signalling and functions as a reporter of its activity (Veeman et al., 2003). As a result, I was able to observe fluorescence in larvae developing from injected embryos.

9.3. Isolation of *Pdu-Tcf*

9.3.1. Preparation of cDNA

I isolated mRNA from various larval stages with TRIzol™ Reagent (Invitrogen 15596026) according to manufacturer's instructions. cDNA was synthesized using Super-Script™ II RT (Invitrogen 18064014) and random hexamer primers (Invitrogen 48190-011) following the manufacturer's protocol.

9.3.2. Cloning

We searched *P. dumerilii* EST databases with sequences of *Tcf* homologues known from other organisms. We found two contigs, 05083 and 02618, that exhibit a high similarity to the β-catenin or HMG-binding domains of *Tcf*, respectively.

By PCR amplification, I obtained a full length C-clamp (-) *Pdu-Tcf* cDNA. I used TcfPlatyRT-1 (5'-GGGAGATTTTCATGGCGGATTCA-3') forward primer together with TcfPlatyRT-4 (5'-CAGTTAGATCAAGCAGAGGTCAGAAGTAATACC-3') reverse primer and Long PCR Enzyme Mix (Thermo Fisher Scientific #K0182) on mixed stage *Platynereis* cDNA as a template. Condition for PCR were as follows: initial denaturation 95 °C/2 minutes followed by 30 cycles of 95 °C/20 seconds denaturation, 61 °C/30 seconds annealing and at 68 °C/3 minutes of extension, after the last cycle closed by an additional 10-minute extension period.

The resulting fragment has been then cloned into pGEM T-Easy Vector System (Promega), taking advantage of A overhangs produced by Long PCR polymerase and corresponding T overhangs in the vector creating cohesive ends. The ligation product was transformed into TOP10 competent strain of *Escherichia coli*, which were cultivated on LB plates with ampicillin antibiotics. The presence of cloned *Pdu-Tcf* was confirmed by restriction digest and sequenced by Sanger sequencing with M13 Universal and Reverse (-20) primers.

9.3.3. Identification of *Tcf* variants

I found by sequencing that *Tcf*, which I have previously isolated, possess a termination codon after the HMG DBD and as a consequence lacks a C-terminal accessory DNA-binding C-clamp domain. This domain is present in all of the protostomes to which

Platynereis belongs and most of the protostomes have only one Tcf gene (Archbold et al., 2012; Cadigan and Waterman, 2012). We thus further searched TSA databases and yielded two more cDNA sequences, GBZT01001652.1 and GBZT01006558.1, from BioProject PRJNA271451 (Yanai, 2015). It is of note that the former possessed a C-clamp and the latter a difference in the beginning of the HMG domain, but otherwise were found to be very similar. I designed two different forward primers using the Primer3 online tool (Koressaar and Remm, 2007; Untergasser et al., 2012) that were specific for the two variants of the 5'-HMG exon and a common reverse primer (Table 1) specific to the very end of C-clamp (+) ORF. I then tried to amplify the C-clamp (+) C-terminus in two separate PCR reactions with AccuPrime™ *Pfx* SuperMix (Invitrogen Life Technologies Cat. No. 12344-040) and succeeded with both primer combinations. Conditions of PCR were the same as for amplification of gene fragments to generate probes for *in situ* hybridization (see further in the chapter 9.8.1). The PCR products were separated by agarose gel electrophoresis resulting in 3 fragments of different sizes for each primer combination, corresponding to 6 different *Pdu-Tcf* cDNAs.

Fragments of different sizes were excised from the gel and isolated by QIAEX II Gel Extraction Kit (Qiagen, 20021) and cloned into a pCR™-Blunt vector using a Zero Blunt™ PCR Cloning Kit (Invitrogen – Thermo Fisher Scientific K2750-20). The cloned fragments were sequenced and used for probe synthesis for *in situ* hybridization (see further).

9.3.4. Intronic analysis

I used the same combination of primers as for isolating C-terminal cDNA variants with *Platynereis* genomic DNA as a template and extended time for synthesis to find the source of this variability, but I did not obtain any product. I thus used a forward primer 5'-AGCGCTCGTGACAATTACGC-3' which anneals to the end of HMG (same in both HMG variants) and a common reverse primer 5'-TCATAGTGGCGGTGGTTCCA-3' for the end of the C-clamp (+) variants (Table 1) on the *Platynereis* genomic DNA template. I used the Long PCR reaction mixture and prolonged time for synthesis, otherwise the conditions for PCR were the same as for the isolation of cDNA (chapter 9.3.2). This procedure yielded one DNA fragment which was sequenced and the sequence compared with previously found cDNAs to identify exon-intron boundaries.

I also tried to reveal the exon-intron structure of the N-terminus of *Pdu-Tcf* (β-catenin binding domain) to verify that CRISPR gRNAs (see further) are not on exon-intron boundaries. I used the 5'-TGCCGCATGTGAACTCAAGCG-3' forward primer complementary to the N-terminus of protein coding region of *Pdu-Tcf* ORF starting with ATG codon (underlined) and a 5'-GCCAATGGTGCTTCACTGGT-3' reverse primer so the resulting product spanned both designed CRISPR gRNAs target sites. This combination of primers was used in a PCR reaction with a *Platynereis* genomic DNA as a template with AccuPrime™ *Pfx* polymerase and the yielded fragment has been cloned into the pCR™-Blunt vector, sequenced and compared to the *Pdu-Tcf* cDNA sequence.

There is a short 36nt sequence that corresponds to 12 amino acids, which is only facultatively included into the transcript, after the second intron of the β-catenin binding domain. It is present in both available cDNA sequences from the closely related polychaete, Perinereis nuntia [NCBI GenBank accession numbers AB701688 and AB701687, (Niwa et al., 2013)], and two *P. dumerilii* TSA sequences but not in another publicly available *P. dumerilii Tcf* sequence (NCBI GenBank number KT266551, Simon F., unpublished). No such sequence was found in other protostome sequences that I analysed and an entirely different sequence was observed in deuterostomes. I thus used the N-terminal probe which excluded this variable region to assess the *Pdu-Tcf* expression patterns although I included this region in the phylogenetic analysis.

9.3.5. Phylogenetic analysis

9.3.5.1. Protein alignment

Tcf/LEF protein alignment was done using BioEdit‘s (Hall, 1999) ClustalW Multiple alignment function, MEGA7‘s (Kumar et al., 2016) MUSCLE algorithms and improved manually. The GenBank accession numbers of protein or translated nucleotide sequences used for comparison are as follows: *Perinereis nuntia* (AB701688.1), *Lingula anatina* (XP_013385963.1), *Crassostrea gigas* (XP_019923475.1), *Biomphalaria glabrata* (XP_013060932.1), *Limulus polyphemus* (XP_022255329.1), *Parasteatoda tepidariorum* (XP_021000199.1), *Drosophila melanogaster* (NP_001033798.1), *Tribolium castaneum* (XP_008191151.1), *Strongylocentrotus purpuratus* (NP_999640.1), *Sacoglossus kowalevskii* (XP_006811841.1), *Branchiostoma floridae* (AAZ77711.1), *Danio rerio* (NP_571334.1), *Xenopus laevis* (XP_018082716.1), *Anolis carolinensis* (XP_008112949.1), *Gallus gallus* (XP_015144041.1), *Homo sapiens* (NP_001185456.1). Pdu-Tcf protein sequence was an *in silico* translation of the longest *Pdu-Tcf* isoform (C-terminal isoform X1) whose full sequence has been obtained by merging the X1 C-terminal sequence with the full length cDNA of the C-clamp (-) isoform X7.

9.3.5.2. Molecular phylogeny

Molecular phylogeny was determined using MEGA7 software (Kumar et al., 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories + G, parameter = 0.8124). All positions with less than 85% site coverage were eliminated. That is, up to 15% alignment gaps, missing data, and ambiguous bases were allowed at any position. The purpose of this was to include the whole HMG domain in the analysis, although two out of the 17 sequences (*Perinereis nuntia* and *Limulus polyphemus*) were truncated and did not contain complete HMG domain. As a result, there were a total of 343 positions in the final dataset.

9.4. Wnt/β-catenin pathway manipulation

9.4.1. Pharmacological treatments

The workflow of pharmacological activation and inhibition of Wnt signalling and sample processing is depicted in the Figure 4. I concentrated larvae of a desired stage (24 hpf, 48 hpf, 5 dpf) from a single batch on a fine mesh sieve and collected them using a Pasteur pipette. The whole batch was then distributed equally to the whole volume by pipetting up and down and divided into several experimental groups (one for control and one for each of the inhibitors). Each group consisted of 4 ml of NSW with larvae on a 6-well plate (for 24 and 48 hpf larvae) or 7 ml in a 6 cm diameter Petri dish (for 5 dpf larvae).

Chemicals used for treatment were dissolved and stored in dimethyl sulfoxide (DMSO, Sigma) at 100 mM as stock solutions and at 6 or 10 µM concentration as a working solutions. The inhibitors in DMSO or corresponding amount of DMSO alone as a control were diluted in a volume of NSW to 1 ml in total and mixed with the larvae. I used CHIR99021 as an activator of the Wnt/β-catenin pathway (Biomedica) at 10µM final concentration, since higher concentrations often killed the larvae, and inhibitors JW55, JW74, OD270 (all three from our own stock, but some of them also available commercially) or IWR-1-endo (Merck Millipore 681669) at a 30µM concentration, because 40µM concentration was used previously (Demilly et al., 2013) but I saw the same effect on gene expression even with lower concentration. The larvae were then incubated at 18 °C and collected at desired stage (48 hpf, 72 hpf, 7 dpf) for Western blotting or fixed for whole mount staining (Figure 4).

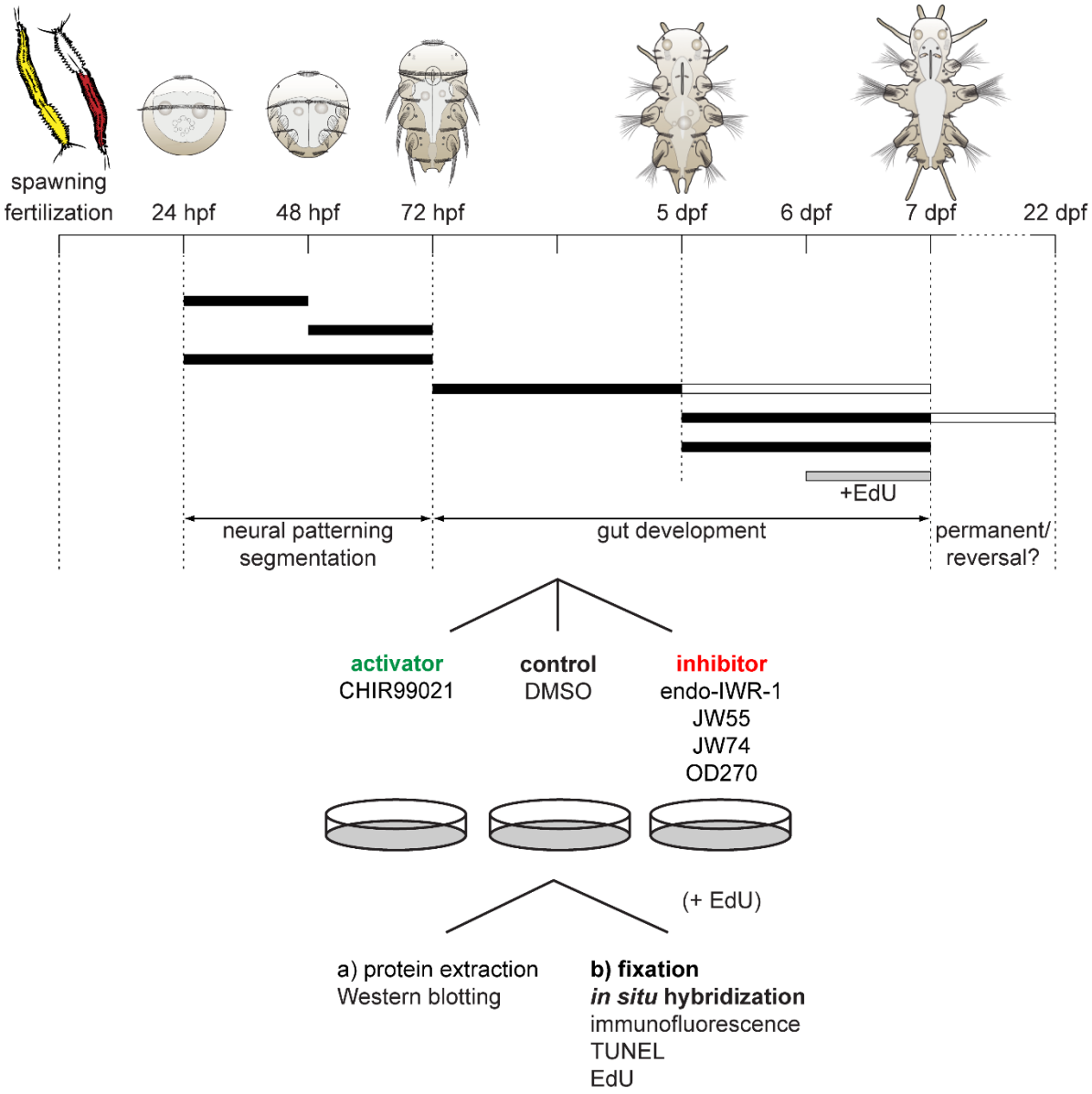


Figure 4 – The time course and the workflow of pharmacological treatments

The main developmental larval stages used in this work are marked on the timescale (in hpf or dpf) with the time windows when the pharmacological treatments with chemical modulators of Wnt/β-catenin pathway were done and the major developmental processes in which the Wnt/β-catenin signalling was investigated. The workflow after chemical treatments (in a 24-well plate on in a 6cm Petri dish, depending on stage) is also outlined (more detailed in the text).

9.4.2. CRISPR/Cas9 knock-out

Small guide RNAs (sgRNAs) were designed using ZiFiT online tool (Sander et al., 2010) and the sequence of first 1000 bp of *Pdu-Tcf* (GenBank accession number MG952772.1) or *Pdu-β-catenin* (GenBank EF581779.1) cDNA. Synthesized DNA oligo-nucleotides coding for sgRNAs were cloned into the CRISPR small guide RNA backbone of pT7-gRNA plasmid digested by BsmBI at 55 °C. Complementary oligonucleotides were first annealed by diluting them in the nuclease free water and a restriction buffer to 20 pmol/µl concentration, preheating the mixture to 95 °C for 5 minutes and them cooling it on ice for 10 minutes. The annealed oligonucleotides were then diluted 10-fold and 100-fold in water and 1 µl was used in a 20 µl ligation reaction. After purification, the constructs were verified by sequencing, linearized by BamHI restriction endonuclease and the sgRNAs were synthesized *in vitro* by MEGAshortscript T7 Transcription Kit (Ambion, AM 1354) according to the manufacturer's protocol. The concentration was measured by NanoDrop spectrophotometer and Qubit™ RNA Assay Kit.

The *Cas9* mRNA was synthesized *in vitro* using mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen™) and MLM3613, linearized by XhoI restriction endonuclease, as a template. Resulting mRNA was checked by electrophoresis on an agarose gel and the concentration measured by Qubit™ RNA Assay Kit.

Cas9 mRNA was mixed with two or three sgRNAs for *Pdu-Tcf* and *Pdu-β-catenin*, respectively. Various concentrations have been tested, ranging from 200µM to 300µM *Cas9* mRNA and 25-100µM of each sgRNA. The mixture was injected into fertilized eggs. Some embryos were harvested for genotyping at 24 hpf, the others were kept until adulthood and genotyped later from amputated tails. DNA from whole larvae or amputated tails was extracted with proteinase K for 2 hours at 55 or 65 °C followed by enzyme inactivation at 80 °C for 25 minutes. Genotyping was done by PCR with DreamTaq Green PCR Master Mix (Thermo Scientific™, K1081), programme: 94 °C/1 minute, 35 cycles of 94 °C/30 seconds, 60 °C/30 seconds, 72 °C/30 seconds and closed by 3 minutes at 72 °C final synthesis. However, after many rounds of CRISPR injections and genotyping, I did not obtain any undisputedly positive results.

9.5. Western blotting

9.5.1. Protein isolation and concentration measurement

Larvae after chemical treatment were first sedated with 1M $MgCl_2$, added to the final 50mM concentration, and then collected with a pipette to a microcentrifuge tube. As much liquid as possible was discarded and replaced by distilled water to the 50 µl final volume. I added 45 µl of Laemmli buffer without mercaptoethanol (for 10 ml: 2 ml sodium dodecyl sulfate, 2 ml glycerol, 1.25 ml 1M Tris pH=6.8, 3.75 dH_2O) to the samples and boiled them for 4 minutes at 95 °C. After this, samples were kept on ice and 20 µl were used to measure their concentration of proteins by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific 23225) according to manufacturer's instructions. Now, 1/20 of remaining volume of mercaptoethanol could be added and the samples incubated 1 more minute at 95 °C and stained by trace amount of bromphenol blue. Later, the measured concentration was used to calculate the amount of sample for Western blots to achieve equal loading.

9.5.2. Western blotting

Proteins were separated by SDS-PAAGE on 3% polyacrylamide stacking gel followed by 7,5% running gel and transferred to PVDF membrane (Bio-Rad #1620177) by wet blotting in methanol-containing transfer buffer (192 mM glycine, 25 mM Tris and 20% MetOH in dH_2O) for 1 hour at 100 V. Membranes were then blocked for 1 hour in 5% milk in PTw, cut in halves with larger and smaller proteins. Parts with larger proteins were incubated with anti-β-catenin (1:100, Sigma C2206) and the latter with HRP conjugated anti-β-actin antibody (Sigma) at 4 °C overnight, rotating.

Next day, membrane incubated with anti-β-catenin primary antibody was washed three times by PTw and incubated with Anti-Rabbit IgG (whole molecule)-Peroxidase (Sigma A6154) secondary antibody, 1:5000 in 5% milk/PTw for 2 hours at room temperature. Both parts of membranes were washed three times in PTw, assembled together and the luminescence developed for 5 minutes in the dark using West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific 34580). The membranes with developed signal were exposed on an X-ray film (Fujifilm).

9.6. Sample collection and fixation

Larvae of the desired stage were immobilized by the addition of 1/10 - 1/3 volume of 4% PFA (prepared from the aqueous solution of 16% PFA, Electron Microscopy Sciences 50-980-487) in PTw (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl, 0.1% Tween20, pH=7.4) and collected by a pipette into a 2ml microtube. After the larvae sedimented to the bottom, the solution was discarded, replaced with 2 ml of 4% PFA/PTw and the samples incubated at room temperature, rocking slowly. After two hours, they were washed three times with PTw and dehydrated with 2 washes in 100% MetOH. Subsequently, fixed larvae were stored in 100% MetOH at -20 °C. All solutions were prepared using diethyl-pyrocarbonate (DEPC, Sigma D5758), treated (1:1000) autoclaved de-ionized water and filtered through a 0.22 µm syringe filters (Merck Millipore SLGS033SS or SLGP033RS) or tissue culture filters (Corning 431097) prior to use.

9.7. Immunofluorescence

9.7.1. Antibody staining

Whole mount immunofluorescence staining was based on *in situ* hybridization protocol. Fixation, storage, re-hydration, permeabilization by proteinase K and subsequent washes with PTw were performed in the microtubes and in the same manner as for the *in situ* hybridization protocol (see below). Unlike the *in situ* hybridization protocol, these steps were followed by blocking in Blocking 1 buffer (Schneider and Bowerman, 2007) and incubation with monoclonal anti-acetylated tubulin (1:1,000, Sigma T 6793) to mark cilia and nervous system and anti-β-catenin (1:100, Sigma C2206) primary antibodies in Blocking 1 at 4 °C, overnight, shaking on a nutating mixer.

On the second day, the larvae were washed 3×15 minutes and 4×30 minutes in PTw and incubated with Alexa Fluor® 555 goat anti-mouse IgG (H+L) (1:500, Molecular Probes A21422) and Alexa Fluor® 647 goat anti-rabbit IgG (H+L) (1:500, Molecular Probes 21245) or Alexa Fluor® 488 goat-anti-rabbit (1:500, Molecular Probes A11034) with DAPI (1:1000) in Blocking 1, overnight in the dark at 4 °C, shaking on a nutating mixer. Unbound or weakly bound antibodies were removed with several washes with PTw and the larvae were transferred via a series of gradually increasing concentrations of 2,2'-Thiodiethanol (TDE; Sigma 166782) in PTw to 97% TDE and stored at 4 °C in the dark.

9.7.2. Phalloidin staining

Muscle actin of 7 dpf larvae was stained with Alexa Fluor 488 conjugated phalloidin (Molecular Probes A12379) as described by Williams et al. (2015), counterstained with DAPI and mounted in 87% glycerol in PTw.

9.8. Probe preparation

9.8.1. Cloning of cDNA

I used mixed stage *Platynereis* cDNA as a template and gene specific primers (Table 1), designed with Primer3 online tool, to amplify cDNA fragments by polymerase chain reaction with AccuPrime™ *Pfx* SuperMix (Invitrogen Life Technologies Cat. No. 12344-040). Conditions of PCR were as follows: initial denaturation 95 °C/5 minutes, 35 cycles of 95 °C/15 seconds denaturation, 61 °C/30 seconds annealing of primers, 68 °C/0:30 to 1:30 min. for synthesis, depending on the size of the expected size of the product.

PCR products were separated on 1% agarose gel in TAE. The band of proper size was excised and the DNA extracted by Qiagen QIAEX II Gel Extraction Kit or Macherey –Nagel NucleoSpin Gel and PCR Clean-up kits. The concentration of isolated PCR products was determined by measurement of DNA absorbance with NanoDrop microvolume spectrophotometer (ThermoFisher Scientific). I cloned the fragments into a pCR™-Blunt vector using a Zero Blunt™ PCR Cloning Kit (Invitrogen – Thermo Fisher Scientific K2750-20) and transformed into TOP10 *Escherichia coli* competent bacterial strain (15-30 minutes on ice, then 35 second heat shock at 42 °C). pCR™-Blunt cloning vector contains gene for kanamycin resistance so I first incubated the transformed bacteria in 1 ml of pure LB medium at 37 °C, rotating 800 rpm in order to allow the bacteria to acquire resistance. I then plated the bacteria on LB plates supplemented with kanamycin and let them grow until the next day. I picked randomly several (usually five) colonies and inoculated them into 2,5 ml of LB medium with kanamycin in a 15ml test tube and grew the culture overnight at 37 °C, rotating 200-250 rpm. I isolated the plasmid DNA with GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific K0503) and used restriction digest to determine the orientation of cDNA fragments in the plasmid by checking the size of digestion products on the TAE agarose gel by DNA electrophoresis.

The *Pdu-Tcf* N- and C-terminal (isoform X7) probes were acquired by PCR with Pfu polymerase with a 48 hpf stage *Platynereis* cDNA as a template. Forward primers (Table 1) had an attached tail that contained the EcoRI restriction site and the reverse primers (Table 1) contained a tail with the BamHI site. The included restriction sites were digested by corresponding digestive enzymes, EcoRI and BamHI, and the resulting complementary ends used to ligate the fragments into the pBlueskript II KS.

Fragments of *Pdu-Tcf* C-terminal isoforms X1-X3 or X4 and X5 were obtained as described (chapter 9.3.3), excised as isolated bands from the electrophoretic agarose gel, extracted and cloned into pCR™-Blunt plasmid.

9.8.2. Probe synthesis

Plasmid templates were linearized with the appropriate restriction enzyme (Table 2), purified with QIAprep Spin Miniprep Kit (Qiagen) and used to generate digoxigenin (DIG)-labelled antisense RNA probes by DIG RNA Labeling Mix (Roche 1277073) following the manufacturer's recommendation. Table 2 summarizes the restriction enzymes and RNA polymerases (Roche) used to synthesize antisense probes together with references of the source of original plasmid (sent by one of the authors) or of the sequences used as a source of information to design own primers. The synthesized probes were diluted in deionized distilled water and their concentration measured using the Qubit™ RNA Assay Kit. The probes were then stored at -80 °C as stock solution or diluted to 2 ng/µl concentration in HybMix (see further in the chapter 5.8.1) as a working solution and stored at -80 or -20 °C.

Table 1 – Primer sequences for probe synthesis

Gene	Forward primer	Reverse primer
Axin	AGTTCCTCAATGACTCGGCA	CTTCCTGTAACGTGGGGAGT
Engrailed	AACGCAGGGAAGAAAGCATG	TTGTACAGTCCTTGGGCCAT
Gsx	GACTTCATCAGCTGCCTTGG	ACTCGCGTTCTAGTTCCAGT
Pax2/5/8	TGGCTGAAGGAGTGTGTGAT	AAGCAGGGAGGGTGTAAGAC
Six3	GCCCCGTCGATAAGTACAGA	CTAGTCAGTGAGAGGGGCTG
Tcf N-terminus	GGGAGATTTTCATGGCGGATTCA	CTGTACAAGGGATGATGGAACTGGC
Tcf C-terminus, isoform X1	TGATGAGAACGAGGTGCAGGA	TCATAGTGGCGGTGGTTCCA
Tcf C-terminus, isoform X2		
Tcf C-terminus, isoform X3		
Tcf C-terminus, isoform X4	GACCACACACCCAATGATAGCG	
Tcf C-terminus, isoform X5		
Tcf C-terminus, isoform X7		CAGTTAGATCAAGCAGAGGTCAGAAGTAATACC
WntA	GCGGGGATTCAGTTCGATTC	GTAGTAACCGCGACCACAAC
Wnt5	GACAAGTACGACGGAGCAAC	TGCGAGACTGCCACATTCTA
Wnt16	ATGCAATCCTCTGTGTGGGA	CGGTCCCTTTGAGTCCTTCT

Table 2 – Plasmid constructs for probe synthesis

Gene	**Vector**	**Orientation** (polymerase for synthesis of an antisense probe)	**Restriction enzyme** (for linearized template of an antisense probe)	**Reference/source**
α-Amylase	pCMV-Sport6	T7	SmaI	(Williams et al., 2015)
Axin	pCR-BluntII TOPO	SP6	NotI	this work
Cdx	pCRII TOPO	T7	EcoRI	(Hui et al., 2009)
Engrailed	pCR-BluntII TOPO	T7	BamHI	?
Emx	pCRII TOPO	SP6	XhoI	(Tomer et al., 2010)
Enteropeptidase	pCMV-Sport6	T7	SmaI	(Williams et al., 2015)
Gsx	pCR-BluntII TOPO	SP6	NotI	this work
Hox1	pCRII TOPO	SP6	EcoRV	?
Hox4	pGEM T-Easy	SP6	SphI	?
Legumain	pCMV-Sport6	T7	SmaI	(Williams et al., 2015)
Lox5	pGEM T-Easy (?)	SP6	SacI	?
Ngn2	pCMV-Sport6	T7	EcoRV	(Simionato et al., 2008)
Nk2.1	pCMV-Sport6	T7	SalI	(Raible et al., 2005)
Otx	?	T7	HindIII	(Arendt et al., 2001)
Pax2/5/8	pCR-BluntII TOPO	T7	BamHI	this work
Pax3/7		T7	HindIII	(Denes et al., 2007)
Pax6		T7	NotI	(Arendt et al., 2002)
Six3	pCR-BluntII TOPO	SP6	EcoRV	this work
Subtilisin-1	pCMV-Sport6	T7	SmaI	(Williams et al., 2015)
Subtilisin-2	pCMV-Sport6	T7	SmaI	(Williams et al., 2015)
Tcf N-terminus	pBluescript II KS	T7	EcoRI	this work
Tcf C-terminus, isoform X1	pCR-BluntII TOPO	SP6	NotI	this work
Tcf C-terminus, isoform X2	pCR-BluntII TOPO	SP6	NotI	this work
Tcf C-terminus, isoform X3	pCR-BluntII TOPO	SP6	NotI	this work

Tcf C-terminus, isoform X4	pCR-BluntII TOPO	SP6	NotI	this work
Tcf C-terminus, isoform X5	pCR-BluntII TOPO	SP6	NotI	this work
Tcf C-terminus, isoform X7	pBluescript II KS	T7	EcoRI	this work
WntA	pCR-BluntII TOPO	SP6	NotI	(Prud'homme et al., 2002) this work
Wnt5	pCR-BluntII TOPO	SP6	NotI	(Janssen et al., 2010) this work
Wnt16	pCR-BluntII TOPO	SP6	NotI	(Janssen et al., 2010) this work

(continuation from the previous page)

9.9. Visualization of gene expression

9.9.1. *In situ* hybridization on larvae

Visualization of gene expression by whole mount *in situ* hybridization was done according to previously published protocols with some minor modifications. Fixed larvae dehydrated in 100% MetOH and stored at -20 °C were rehydrated by subsequent 5 minute washes in decreasing dilution series (75 %, 50 % and 25 %) of MetOH in PTw (DEPC treated, filtered) at room temperature. The samples were left still on the table between washes to allow larvae to settle to the bottom of the tubes so the solution could be readily aspired without checking under the microscope. After 3 washes in PTw, the larval cuticle has been permeabilized by freshly prepared 0.1 mg/ml proteinase K in PTw. The lengths of the incubation were as follows: 1 minute for 24, 48 and 72 hpf larvae, 2-2.5 min. for 5 dpf larvae and 2.5-3 min. for 7 dpf larvae. The activity of proteinase K was stopped by 2 washes (each corresponding in length to the time of proteinase K treatment) in 2mg/ml glycine in PTw (prepared in advance and stored at -20 °C in aliquots sufficient for several *in situ* hybridization experiments). The larvae were then re-fixed by 4% PFA/PTw for 30 minutes at room temperature, slowly rocking. The fixative was then removed by 5 washes in PTw (at least 5 minutes per each wash, without rocking). Larvae were then transferred to hybridization buffer [HybMix: 50% de-ionized formamide, 5× SSC (3 M NaCl and 0,3 M sodium citrate in DEPC-treated deionized distilled water, pH=4.5), heparin 50 µg/ml, 0.25% Tween20, 1% sodium dodecyl sulfate and 50 µg/ml single stranded DNA from salmon testes (Sigma D9156) in DEPC-treated de-ionized water] by one 10-minute wash in 150 µl of HybMix. After the transfer to fresh hybridization buffer, larvae from one experimental group (one treatment, one stage) were divided into several samples, 150 µl of HybMix per sample, for staining of different genes. The samples were pre-hybridized at 63 °C in a thermal block with a cover. After 2-3 hours, the pre-hybridization solution was replaced with 50 µl of HybMix with 2 ng/µl digoxigenin-labelled RNA probes, which were denatured previously for 10 minutes at 90 °C. The samples were hybridized approximately for 16-18 hours overnight in a thermal block at 63 °C.

The next day, probes were collected and were stored at -80 or -20 °C and could be re-used up to five times. The samples were still kept at 63 °C and washed with 250 µl of plain hybridization buffer pre-heated to the same temperature in water bath. After 10-15 minutes, when the larvae settled to the bottom of the tubes, the solution was aspired by a

pipette and replaced by another 15-minute wash with 250 µl of HybMix or 0.5 ml of 2× SSCT/50% formamide (Sigma). SSCT solutions were prepared from 20× SSC (3 M NaCl and 0,3 M sodium citrate in DEPC-treated deionized distilled water, pH=7) and 20% Tween20 in DEPC-treated deionized distilled water. Subsequently, the samples were washed twice in 2× SSCT/50% formamide, 30 minutes per wash, twice in 2× SSCT, 15 minutes per wash, and twice in 0.2× SSCT, 30 minutes per wash. All steps were done with the samples kept in a thermal block at 63 °C, the solutions prepared with DEPC treated autoclaved de-ionized water and pre-heated to the hybridization temperature. The solutions were exchanged without the samples leaving the thermal block relying on the sedimentation of the larvae to the bottom of the tubes. Most of the solution was aspired with only 20-50 µl left. After last SSCT wash, the samples were transferred back to room temperature and washed twice with RT/cold 1× PTw and blocked in 2 % w/V Boehringer-Mannheim Blocking Reagent (Roche 11 096 176 001) in maleic acid buffer (0.1M maleic acid, 0.05M NaCl, pH = 7.5), supplemented with 0.01% Tween20 (MABT), for 1 hour at RT, slowly rocking. The larvae were then incubated with anti-Digoxigenin-AP, Fab fragments from sheep (1:4,000, Roche 11 093 274 910) and monoclonal anti-acetylated tubulin (1:1,000, Sigma T 6793; only applied for fluorescent *in situ* hybridization protocol) antibodies in 2 % w/V Blocking/MABT, shaking on a nutating mixer at 4 °C overnight.

On the third day, the samples were washed with PTw, three times 5-10 minutes and four times 30 minutes per wash. After the last wash, the larvae were stored overnight in the fridge or nutating in the cold room at 4 °C in PTw. The next morning, the samples were washed briefly (5-10 minutes) three times with PTw before I proceeded to staining.

For fluorescent *in situ* hybridization, the samples were washed twice with 100mM Tris-Cl, pH=8.5, 0.2% Tween20, which had been previously filtered through a 0.22 µm syringe filters (Merck Millipore). The gene expression signal was developed by the incubation of larvae with the Vector® Blue Alkaline Phosphatase Substrate (Vector Laboratories SK-5300) staining solution on 4-well or 24-well plate in the dark at room temperature, slowly rocking. Vector Blue staining results in a blue precipitate which is visible with bright field microscopy to check the development of signal, yet it provides a strong fluorescent signal (Hollinshead et al., 1998). The staining solution was prepared according to manufacturer's instructions by dilution of three kit components in 100mM Tris-Cl, pH=8.5, 0.2% Tween20.

For bright field microscopy, the larvae were washed twice (10 minutes per each wash) in alkaline phosphatase (AP) staining solution without $MgCl_2$ (50mM Tris-Cl,

pH=9.5, 100mM NaCl, 0.1% Tween20) followed by two washes with the same buffer supplemented with 50mM $MgCl_2$. All solutions were filtered through a 0.22 µm syringe filter (Merck Millipore). The staining was done in the AP staining solution with $MgCl_2$, 1.65 µl NBT (Roche 11 383 213 001) and 1.65 µl BCIP (Roche 11 383 221 001) per ml. Stock solutions of NBT and BCIP were first centrifuged for 2-3 minutes at 14,100 rcf to pellet the precipitated material. A signal was developed in the dark at room temperature, slowly rocking, or overnight at 4 °C in the same solution diluted 2-2.5 times by AP buffer + $MgCl_2$.

In both cases, the larvae were checked regularly for a developing signal. To stop the staining, the larvae were transferred back to microtubes and washed five times (about 10 minutes each) in PTw.

For fluorescent *in situ*, larvae were blocked for 1 hour at RT, slowly rocking, in a Blocking 1 solution (Schneider and Bowerman, 2007) (4% sheep serum, 2 mg/ml BSA and 0.1% DMSO in PTw). The samples were then incubated overnight at 4 °C on nutating mixer in Blocking 1 solution containing DAPI (4′,6-diamidino-2-phenylindole, Sigma, 1:1,000) and Alexa Fluor® 555 goat anti-mouse IgG (H+L) (1:500, Molecular Probes. A21422) secondary antibody. The secondary antibody was discarded, followed by two 15 minute and one 30 minute washes in PTw

For both fluorescent and non-fluorescent *in situ* hybridization protocols, the washed larvae were transferred gradually by 10 minute washes through a dilution series of 33%, 66% and 97% (two times) to 97% 2,2'-TDE in PTw. The stained larvae could be stored at 4 °C in the dark for up to several weeks if they were stained with Vector Blue, otherwise the precipitate dissolved in TDE after longer time. The larvae stained with NBT/BCIP could be stored much longer since the precipitate is stable in TDE.

9.9.2. *In situ* hybridization on adult tails

Adult worms were subjected to starvation for 2 days prior to biopsy in order to empty their digestive tracts. They were then immobilized by the addition of 1M $MgCl_2$ to sea water with worms to the final concentration of 50-100mM, which is substantially less than previously reported by others (Ackermann et al., 2005; Fischer et al., 2010; Pfeifer et al., 2012) but still proved to be sufficient. The last 20-25 segments were cut and worms were then put back to the breeding facility, where they regenerated and continued to grow and reproduce normally. The amputated tails were fixed in 4% PFA/PTw (DEPC treated, filtered) overnight at room temperature, slowly rocking. Next day, they were washed three

times in PTw, dehydrated by two washes in methanol and stored in 100% MetOH at -20 °C.

Whole mount *in situ* hybridization was done using the same protocol that was used for larvae with modifications as described in (Pfeifer et al., 2012): proteinase K treatment was prolonged to 10 minutes and the specimens in the pre-hybridization buffer were heated to 80 °C for 30 minutes prior to hybridization in order to inactivate endogenous phosphatase activity. The hybridization was done at 63 °C for 90 hours. Primary antibodies used were anti-Digoxigenin-AP, Fab fragments from sheep (1:4,000, Roche 11 093 274 910) and anti-β-catenin (1:100, Sigma C2206). Fluorescent *in situ* signal was developed by incubation with Vector® Blue followed by staining with DAPI together with Alexa Fluor® 555 goat anti-mouse IgG (H+L) (1:500, Molecular Probes. A21422) secondary antibody in Blocking 1. Stained samples were transferred through increasing dilution series to 97% TDE and stored at 4 °C.

9.9.3. Embedding and sectioning

After *Pdu-Tcf in situ* hybridization with NBT/BCIP on larvae or adult's tails, the samples were washed 1× with distilled water, 1× in 70% EtOH for 30 seconds and in 100% ethanol for 1 minute. The EtOH was replaced for 400 µl of Spurr low viscosity embedding resin (Sigma EM0300, prepared according to the manufacturer's instructions) and incubated for at least 20 minutes while gently rocking. The samples were then placed into moulds filled with Spurr resin and left for 2 hours at room temperature. They were positioned and oriented within the moulds and incubated at 72 °C overnight. Blocks were then sectioned to 4 µm thin sections.

9.10. Cell proliferation and cell death

9.10.1. Cell proliferation

9.10.1.1. EdU labelling

First, I did pharmacological treatment with Wnt/β-catenin pathway activator or inhibitors at 5 dpf as described previously. A day later, 5-ethynyl-2'-deoxyuridine (EdU) from stocks in DMSO or PBS was added to the larvae to a 20μM final concentration. The larvae were incubated in the presence of EdU until 7 dpf when the larvae were fixed by 4 % PFA/PTw and stored in 100% methanol. EdU incorporated into the newly synthesized DNA of proliferating cells was detected using Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Invitrogen, C10339) according to the manufacturer's protocol, followed by staining of nuclei by DAPI, overnight in PTw at 4 °C. After several washes with PTw, the larvae were transferred gradually to 97% TDE mounting medium for confocal fluorescence microscopy.

9.10.1.2. Endogenous alkaline phosphatase staining

Endogenous alkaline phosphatase was detected by Vector Blue phosphatase substrate to visualize the digestive tract at 7 dpf prior to developing EdU signal, similar to *in situ* hybridization procedure omitting the hybridization steps and high temperature to preserve the phosphatase activity. After EdU and DAPI staining and several PTw washes, the larvae were transferred to 97% TDE for fluorescence microscopy.

9.10.2. TUNEL assay

Larvae treated with Wnt/β-catenin pathway activator or inhibitors between day 5 and 7 of development were fixed and stored in 100% methanol. Dead or dying cells were detected using Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Kit (Invitrogen, C10245) and nuclei counterstained with Hoechst 33342 following the kit protocol. After several washes in PTw, the larvae were gradually transferred to 97% TDE for microscopy.

9.11. Data acquisition and processing

9.11.1. Microscopic slide preparation

For both bright field or fluorescent microscopy, the larvae were placed in 80 µl of 97% TDE on a glass slide between two patches, each consisting of 3 layers of Scotch tape as spacers (approximate thickness of each layer is 50 µm) to prevent a deformation of larvae by coverslip. 97% TDE has the same refractive index as glass and low photobleaching (Asadulina et al., 2012) making it an ideal mounting medium for fluorescence microscopy (Staudt et al., 2007).

9.11.2. Confocal microscopy

Immunofluorescent and fluorescent *in situ* hybridization images were taken using Leica TCS SP5 AOBS Tandem microscope with the LP/-/C HC PL APO 40x/1.30 OIL CS2 or LP/0.14-0.20/D HC PL APO 63x/1.40 OIL objective lenses and Leica Application Suite Advanced Fluorescence (LAS AF) software or with Leica TCS SP8 microscope with the APO 40x/1.30 OIL CS2 objective and LAS X software. Images were taken as *z*-stacks with a *z*-step of 0.42 µm and 0.42 µm pixel size, i. e. with cubic voxels, and the appropriate wavelengths for excitation (633 or 635 µm for Vector Blue for Leica SP5 and Leica SP8, respectively) and emission detection (720-800 µm for Vector Blue). I used the *z*-compensation function of laser intensity and detector gain to eliminate the effect of signal loss with increasing depth in a sample. EdU and TUNEL stainings were imaged by Zyla 4.2 PLUS sCMOS camera (Andor) on the Dragonfly 503 spinning-disc confocal system (Andor) mounted on a Leica DMi8 core with HC PL APO 40x/1.30 OIL CS objective lens in Fusion software with 0.45 µm voxel size.

9.11.3. Bright field microscopy

Bright field images and composite images of bright field and fluorescence were taken on a Nikon Diaphot 300 inverted microscope with DIC optics with set Kohler's illumination by Canon EOS1100D camera with the Canon EOS Utility software's Remote Shooting function. Multiple images in different focal planes were taken for a single specimen in the case of whole fixed larvae in order obtain later full focus images.

Photographs of living 21 dpf larvae were taken with Olympus SZX9 stereomicroscope equipped with Olympus DP72 camera and QuickPHOTO micro 2.1 software.

9.11.4. Image processing

Brightness and contrast were adjusted linearly and uniformly for all *z*-stacks from the same experiment (the same stage and gene but different treatment) to allow for comparisons. Maximum *z*-projections (for 7 dpf β-catenin, EdU, AP and TUNEL stainings) or 3D reconstructions (for *in situ* hybridization, phalloidin and 48 hpf β-catenin staining) were done with FIJI software, in the latter case with its 3D Viewer plug-in. Extended depth of field (full focus) images were assembled in Helicon Focus 5 software from several different focal planes of the same specimen. Images were cropped and resized in FastStone Image Browser and the figures assembled in Adobe Illustrator CS4.

9.11.5. Statistical evaluation

For 7dpf stage (gut marker genes), I took a bright field image from every individual after *in situ* hybridization and assigned them to categories according to the expression to quantify the effect of chemical treatment. Proliferating cells marked by EdU incorporation and dead/dying cells marked by TUNEL staining were counted manually on maximal projections of whole *z*-stacks using the Cell Counter plugin in FIJI. The differences between treatments were evaluated by a Student's t-test.

10 Results

10.1. Components and activity of the Wnt/β-catenin pathway

10.1.1. Expression of *Wnt* genes in *Platynereis*

Thanks to predominantly short-range action of Wnt signalling (Clevers and Nusse, 2012), i. e. the signalling is active in the place, where the signal is produced or its close proximity , expression patterns of *Wnt* genes can serve as a good approximation of the site of the Wnt pathway activity.

I cloned and checked by the fluorescent whole mount *in situ* hybridization the expression patterns of three *Wnt* genes, *Pdu-WntA*, *Pdu-Wnt5* (primarily non-canonical Wnt in other organisms) and *Pdu-Wnt16*, at 48 hpf with an original intention to use them as markers of segments. All three *Wnt* genes possess segmental expression (Figure 5 ventral and termino-ventral views) but differ in their exact domains and germ layers in which they are expressed (most apparent on lateral views in the Figure 5). *Pdu-WntA* is present in the ventral-lateral field of the episphere, the site of *Pax6* expression (section 10.2.2.1) and the area of larval eyespots' development (section 7.3.7.1), the stomodaeum and the ventral peristomium and in two adjacent domains of segmental ectoderm and/or the adjacent mesoderm of dorsal and ventral chaetal sacs (corresponding to the future notopodium and neuropodium of parapodia, respectively). *Pdu-Wnt5* is weakly expressed in the lateral hyposphere, segmentally in the ventral neuroectoderm and in the dorsal, but not ventral segmental ectoderm/mesoderm of the chaetal sacs, where the *WntA* transcripts are also present. *Pdu-Wnt16* is expressed only in the hyposphere segmentally in the ventral neuroectoderm and also weakly dorsally.

However, complete expression patterns of all 12 *Pdu-Wnt* genes have been already published for the 48 hpf stage (Pruitt et al., 2014) and provide a good evidence about the overall sites of Wnt activity during neuroectoderm patterning and specification of primary (larval) segments. Looking for the expression of *Pdu-Wnt* genes at later stages, e. g. in the search for a Wnt that could represent a signal for the developing midgut at 7 dpf in *Platynereis*, would require testing of all 12 different *Pdu-Wnt* genes. I found this not to be necessary to determine the function of Wnt/β-catenin in this tissue but would be worth of looking at in the future.

Eventually, I did not test changes in the expression of *Pdu-WntA*, *Pdu-Wnt5* and *Pdu-Wnt16* genes after pharmacological treatments of Wnt/β-catenin pathway as it could not be easily determined whether the potential feedback is direct or indirect via Hh and would require also an inhibition and testing of Hh pathway.

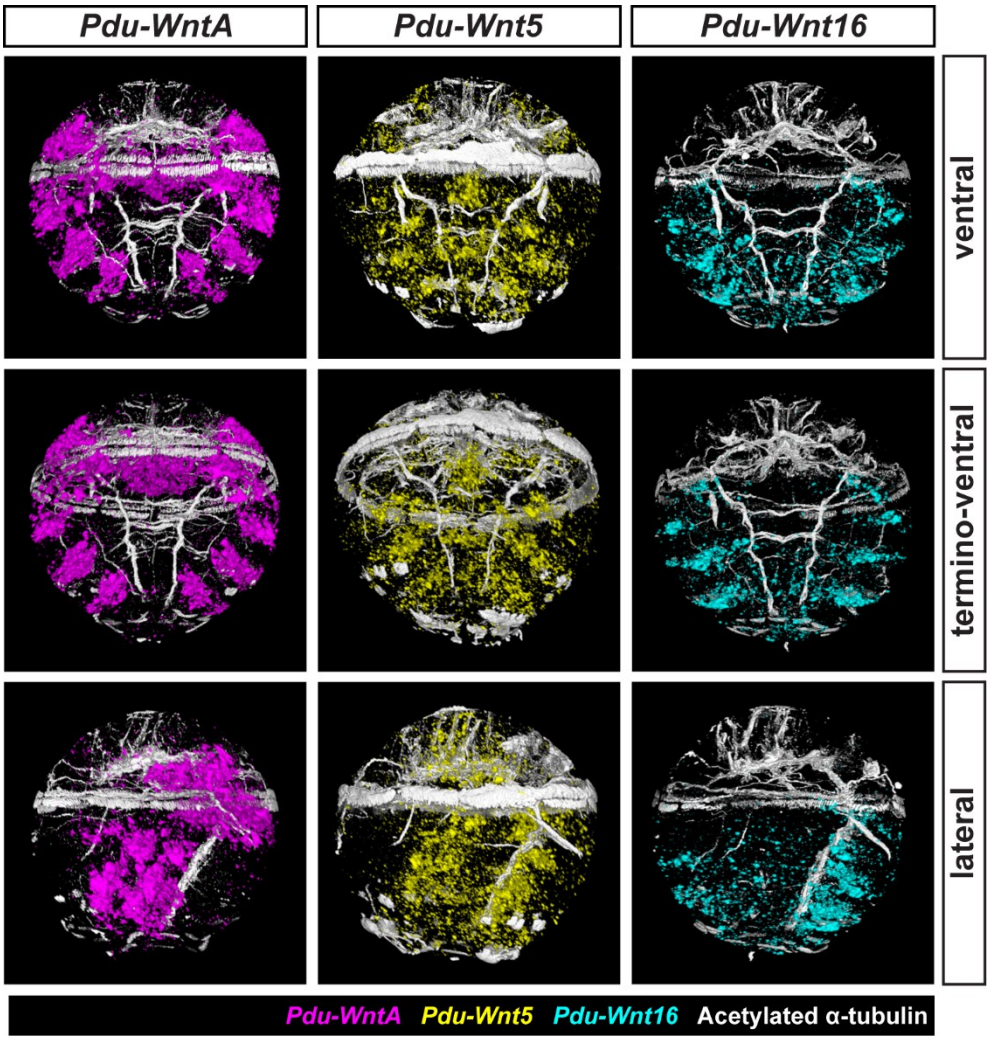


Figure 5 – Expression of representative *Wnt* genes at 48 hpf

All three Wnt genes shown here exhibit pronounced expression in the developing segments, as is apparent from ventral and termino-ventral views, but *Pdu-WntA* seems to be present in the deeper mesodermal layer in chaetal sacs, *Pdu-Wnt16* in the superficial ectodermal layer and *Pdu-Wnt5* in both (as apparent best from the lateral view). Moreover, *Pdu-WntA* and *Pdu-Wnt5* are present in the stomodeal rosette (in the middle below the prototroch) and *Pdu-WntA* also in the lateral regions of the episphere where the larval eyes appear earlier in development.

Approximate size of a 48 hpf larva after *in situ* hybridization procedure is around 130 μm, orientation as indicated - anterior up; in lateral view ventral to the right. Due to a synchronous and stereotypic development of *Platynereis* the staining is highly uniformous in all larvae from the same batch up to 7 dpf stage and only representative individuals can be shown.

10.1.2. *Pdu-Axin* and pharmacological manipulations of the Wnt/β-catenin pathway in *Platynereis*

The expression pattern of *Platynereis Axin* gene has been published before for 33 hpf and 55 hpf stages and it was shown to be downregulated after Wnt/ β-catenin inhibition by IWR-1-endo (Demilly et al., 2013). This suggests that it might be not only a member of the destruction complex of the canonical Wnt pathway, but also its target gene like the *Axin2* in vertebrates (Jho et al., 2002; Lustig et al., 2002) and could be thus used as a readout for Wnt/β-catenin signalling activity for verification of the treatment efficacy.

I cloned my own probe for *Pdu-Axin* with gene-specific primers and used it for *in situ* hybridization on larvae fixed after pharmacological treatments with the activator (CHIR99021) or the inhibitors (JW55 and IWR-1-endo) of Wnt/β-catenin signalling from 24 to 48 hpf or from days 5 to 7 of development. The solvent alone (DMSO) was used for mock treatments in control groups and the results were considered as wild type expression. CHIR99021 inhibits the action of GSK-3β and hence of the destruction complex, which leads to a stabilization of β-catenin and mimics the activation of Wnt/β-catenin signalling by Wnt proteins (Bennett et al., 2002). Either JW55 and IWR-1-endo are inhibitors of tankyrase 1 and tankyrase 2 (Narwal et al., 2012; Waaler et al., 2012) which normally regulate Axin by catalysing its poly(ADP-ribosyl)ation that targets it to the ubiquitylation and degradation (see the section 7.1.4.3). The stabilization of Axin by inhibition of tankyrases occurs on the protein level and hence the chemicals should not affect the *Axin* mRNA directly but only via Wnt target gene transcription. Both inhibitors were shown previously to inhibit Wnt/β-catenin pathway in various systems (Chen et al., 2009; Waaler et al., 2012) and IWR-1-endo was used successfully for this purpose also in *Platynereis* (Demilly et al., 2013).

At 48 hpf stage (Figure 6A), *Pdu-Axin* displays the highest expression in two longitudinal domains that abut the ventral midline (former blastoporal lips), similar to what has been published before for 55 hpf (Demilly et al., 2013), but also in the middle of the stomodeal rosette and the lateral sides (larval eye region) of the episphere and in a lower level in the entire body. Upon the pharmacological activation of the Wnt/β-catenin signalling, *Pdu-Axin* is upregulated in the entire body of the larva except the already differentiated ciliary belt. The inhibition of the Wnt/β-catenin pathway results in the overall lower levels of *Pdu-Axin*. The relatively highest amount of trans can be observed just lateral to the ventral nerve cords, probably also in the blastopore lips which are not fused

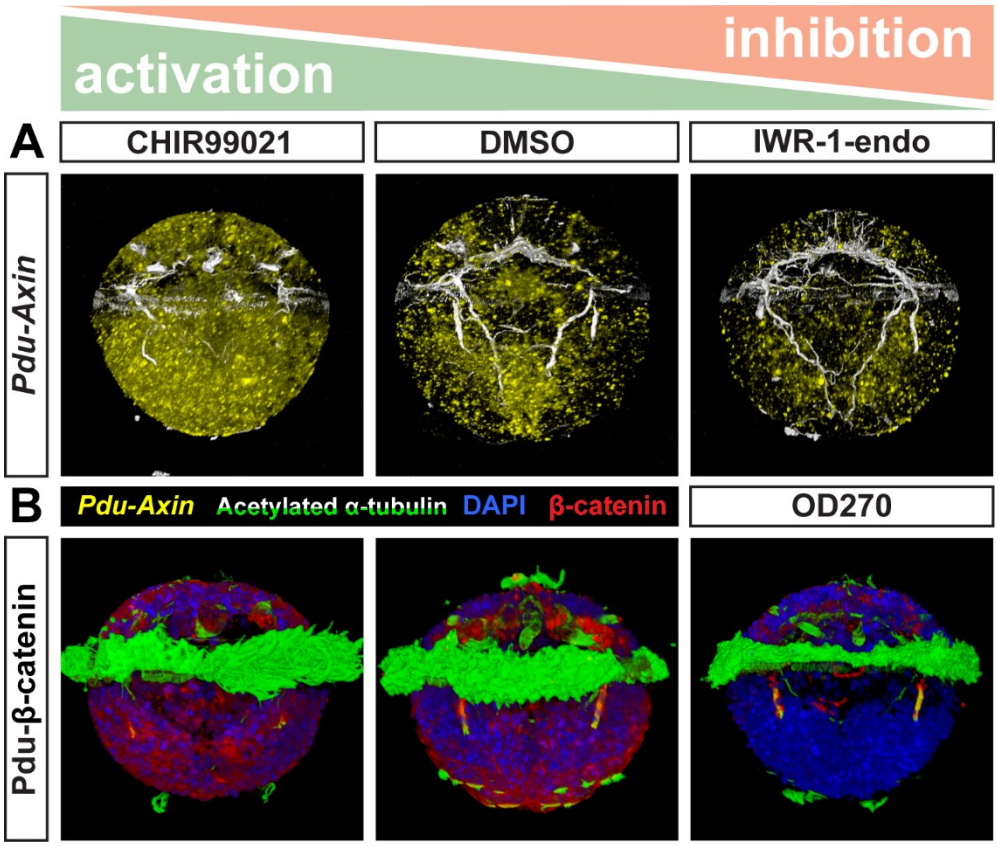


Figure 6 – The effect of pharmacological manipulation of Wnt/β-catenin pathway on the amount and localization of *Pdu-Axin* mRNA and β-catenin protein

(A) *In situ* hybridization of *Pdu-Axin* (*yellow*) at 48 hpf shows almost ubiquitous expression with the peak in the two longitudinal domains along the ventral midline in the hyposphere, as reported previously by (Demilly et al., 2013). After pharmacological treatment from 24 hpf to 48 hpf with Wnt/β-catenin activator (CHIR99021), *Pdu-Axin* transcripts are abundant throughout the body of larva, mainly in the hyposphere. The inhibitor (IWR-1-endo) has the opposite effect, lowering the expression mainly in the regions with otherwise strongest expression. Acetylated α-tubulin (*grey*) represents a stabilized form of tubulin which is present in neuronal axonal projections and in cilia.
(B) Immunofluorescent detection of β-catenin protein (*red*) shows that it is present in highest amount in the episphere, in the developing segments and to a lesser extent also along the ventral midline. After the pharmacological activation of Wnt/β-catenin pathway by CHIR99021 from 24 hpf to 48 hpf, the level of β-catenin is ubiquitously high. When the pathway is inhibited (here by OD270), the overall level of β-catenin is low. More active signalling also means the larger proportion of nuclear (active in signalling) β-catenin as shown by Pearson's correlation coefficients of co-localization between β-catenin and DAPI (nuclei): CHIR99021 (0.45) > DMSO (0.30) > OD270 (0.17). DAPI – *blue*, acetylated α-tubulin – *green*.
Approximate size of a 48 hpf larva is around 130 μm, all images are ventral views with anterior to the top. Representative individuals are shown.

completely, which is reflected also in the absence of most of the commissures between the VNCs.

At 7 dpf (Figure 7A), some transcripts of *Pdu-Axin* are present throughout the body but high expression occurs only in the midgut tissue and in a ring of cells between pygidium and the last larval segment. This ring of *Pdu-Axin* expression is putatively identical to the posterior growth zone (de Rosa et al., 2005; Prud'homme et al., 2003), also known as the segment addition zone (SAZ) (Saudemont et al., 2008), where the proliferation occurs and new segments are added. The activation of Wnt/β-catenin signalling by CHIR99021 did not have any major effect on the *Pdu-Axin*, as it was nor more strongly nor ectopically expressed. This suggests that the CHIR99021 activator for unknown reason does not function in the 5 to 7 dpf time window and/or there are efficient mechanisms that restrict Wnt/β-catenin to certain tissues and whose ability to downregulate Wnt signalling was not overcome by the pharmacological activation. Conversely, the inhibition by either JW55 or IWR-1-endo on average led to overall decreased levels of *Pdu-Axin* transcript. However, there was a high variability in the levels of *Pdu-Axin* expression in all experimental groups and the effect of neither inhibition was not pronounced (Figure 7B).

10.1.3. β-catenin

β-catenin is the sole messenger that transmits the canonical Wnt signal to the nucleus. The nuclear localization of β-catenin is a good indicator of the Wnt/β-catenin pathway's activity. However, to observe nuclear β-catenin is not always possible, because at the same time there is more β-catenin with its second structural role in adhaerens junctions. Nevertheless, these two functions compete for the common pool of β-catenin and the overall amount of β-catenin thus also reflects the activity of Wnt/β-catenin signalling (cf. the section 7.1.4.3). I used a cross-species anti-human/mouse-β-catenin antibody to detect the β-catenin protein in *Platynereis* whole mount larvae after pharmacological treatments of the Wnt/β-catenin pathway and in protein lysates from the treated larvae on the Western blot. This antibody is raised against a C-terminal peptide of human/mouse β-catenin. Based on a protein alignment (not shown), this peptide is not completely conserved between *P.dumerilii* and vertebrates but the antibody has been successfully used before to detect β-catenin in *Platynereis* (Schneider and Bowerman, 2007).

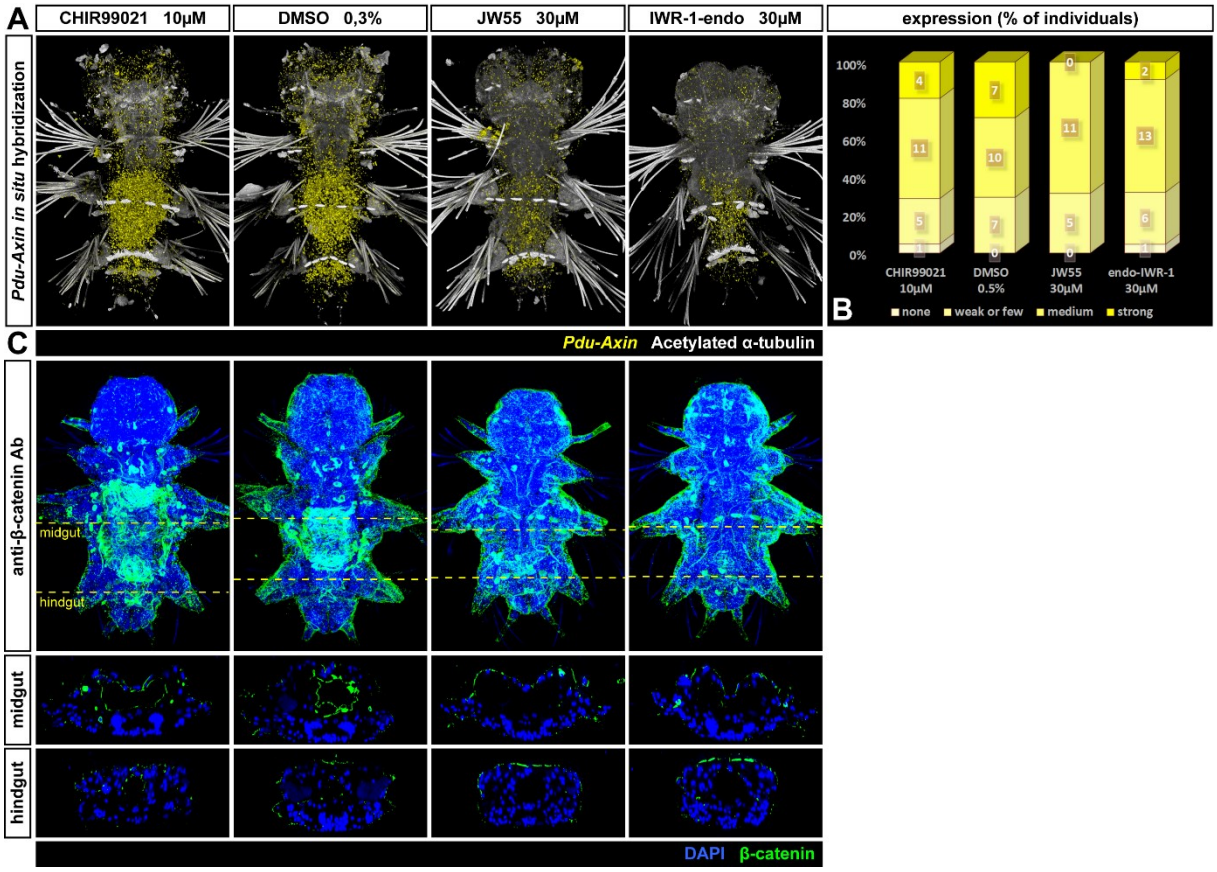


Figure 7 – Components of the Wnt/β-catenin pathway are present in the larval gut and they are affected by pharmacological treatments

(A) 3D projections of confocal *z*-stacks from fluorescent *in situ* hybridization with a probe to detect a putative Wnt target gene *Pdu-Axin* (yellow) and a reference immunofluorescent staining of acetylated α-tubulin *(grey*) on the larvae treated with pharmacological activator (CHIR99021) or inhibitors (JW55 and IWR-1-endo) of Wnt/β-catenin signalling pathway from day 5 to day 7 of development.

(B) Quantification of *Pdu-Axin in situ* hybridization results by the number of individual in each phenotypic class regarding the level of expression. The activator (CHIR99021) does not affect *Pdu-Axin* levels and both Wnt/β-catenin inhibitors display only mild effects on its expression. These larvae come from the same batch as those used for in situ hybridization in the Figure 27 (neurospecific transcription factors in the gut).

(C) Maximal projections (top) and orthogonal virtual sections (below) on the level of midgut and hindgut (indicated by a *dashed yellow line*) of fluorescent confocal *z*-stacks of β-catenin protein immunostaining (*green*) on 7 dpf larvae show that β-catenin is present in the midgut in higher levels than in the hindgut (DMSO control). After pharmacological treatment of Wnt/ β-catenin pathway from 5 to 7 dpf this state persists upon activation. The difference in gut size can be accounted to the variable contraction/dilation of gut smooth muscles upon fixation.

(legend continues on the next page)

(Figure 7 – continuation from the previous page)

On the other hand, the inhibition of the pathway by either of the inhibitors, JW55 or IWR-1-endo, leads to a dramatic drop down in the midgut levels of β-catenin to what appears to be normal for hindgut and the two compartments become indistinguishable regarding the β-catenin staining. The changes in the levels of β-catenin also confirm efficacy of the pharmacological treatments.
The larvae shown here come from the same batch as those used for in situ hybridization in Figure 26 (gut digestive enzymes) and Figure 28 (*Pdu-Cdx*).
All images are dorsal views with anterior to the top. Representative individuals are shown in (C).

In the early metatrochophore developmental stage at 48 hpf (Figure 6B), β-catenin is present in highest levels in the posterior of the larva, in the segmental pattern and medial ventral neuroectoderm and in the episphere. However, a nuclear localization of β-catenin could not be distinguished except in the one control staining. The inhibition of GSK-3β by CHIR99021 leads to an ectopic stabilization of β-catenin in the entire body of the larva and to a higher amount of β-catenin detected on the Western blot (Figure 8).

For the inhibition of Wnt/β-catenin pathway, I tested two more tankyrase inhibitors JW74 (Waaler et al., 2011) and OD270 on the Western blot. When all the inhibitors were used at the same concentration, the most pronounced drop in the amount of β-protein could be seen in the lysates from the larvae treated by IWR-1-endo, followed by the lysates from the JW55 treated larvae. Therefore, I used IWR-1-endo for most of the following experiments and in cases, when I wanted to confirm the results by using another inhibitor, I used JW55. IWR-1-endo usually caused stronger and more reliable inhibition at the same concentration and gave more robust results. JW55 seemed not to work equally well on all batches of larvae, but when it did, it changed the gene expression in the same direction as IWR-1-endo. The effect of JW74 on the Western blot was weak and OD270 seemed not to decrease β-catenin levels at all. In contrast, OD270 caused almost a complete loss of β-catenin staining in whole mount immunofluorescence (Figure 6). The larvae treated with OD270 were tested for *in situ* hybridization with several genes I observed only mild effects on gene expression. I thus soon abandoned OD270 in favour of other inhibitors. I did not use JW74 for its poorer solubility in aqueous solutions and worse results on the Western blot.

At the day 7 of development (Figure 7C), β-catenin is apparent in moderate levels on cellular outlines, which indicates that in most of these tissues β-catenin plays mostly a

structural role and the Wnt signalling is not active here. However, strikingly high amount of β-catenin appears in the midgut, but not hindgut. Together with the observed midgut expression of *Pdu-Axin* (chapter 10.1.2), this suggests that the Wnt/β-catenin pathway could be active in the midgut, where it stabilizes β-catenin and drives the transcription of Wnt target genes. In agreement with this conclusion, β-catenin almost completely diminishes from the midgut of *Platynereis* larvae if they are kept in the sea water treated with either of the inhibitors of Wnt/β-catenin signalling JW55 or IWR-1-endo during the last 2 days of development prior to fixation.

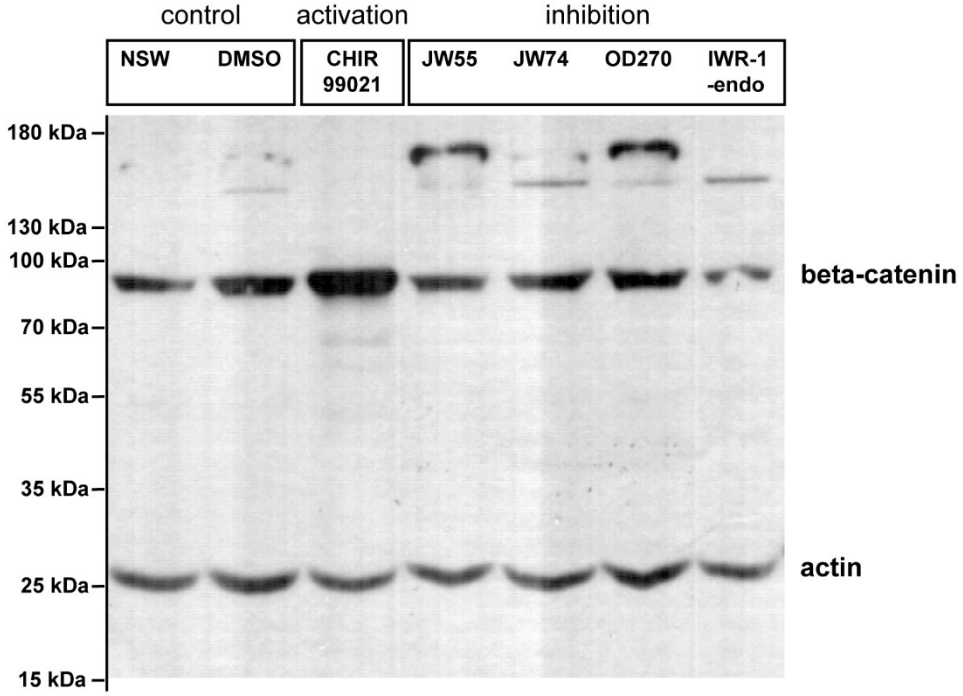


Figure 8 – Shifts in levels of β-catenin after treatments evaluated by Western blot

Changes in the overall levels of β-catenin after pharmacological modulation of Wnt/β-catenin pathway from 24 to 48 hpf were evaluated by Western blotting on protein lysates from 48 hpf larvae. Although the protein concentration in the samples was calculated in advance and the amount used for blotting has been adjusted accordingly, there is some variability in protein loading as revealed by the thickness of actin bands used as a loading control. The activator CHIR99021 shows significant upregulation of β-catenin, whereas IWR-1-endo causes β-catenin amounts to clearly drop down. The results for the remaining three inhibitors, JW55, JW74 and OD270 are less unambiguous – the amount of β-catenin in JW55 is also lowered and this compound was thus used as a second inhibitor in the experiments that investigated the gut development. The loading of OD270 seems to be somewhat higher than for other samples and with respect to the effect of OD270 on β-catenin whole mount immunofluorescence, it was also tested along with IWR-1-endo in some parts of the project.

10.1.4. Transgenic reporter of Wnt/β-catenin pathway's activity

To lend a further support to the idea of the midgut as a major site of canonical Wnt activity by the end of synchronous development in one week old *Platynereis* larvae, I tried to generate a transgenic reporter line that would response to the activity of the Wnt/β-catenin pathway. Such transgenic line could be also used to easily evaluate the efficacy of pharmacological treatments

I used a transgenic construct based on the Mos/Mariner transposons from *Drosophila* which was previously used for stable transgenesis in *Platynereis* (Backfisch et al., 2013). I replaced the part between Mos repeats with a gene for destabilized form of EGFP (to observe the actual pathway activity almost in real time) or LacZ under the SuperTOP promoter which contains an array of 7 Tcf/Lef binding sites (Figure 9A). I then microinjected the resulting transgenic construct together with the mRNA for Mos transposase into the fertilized *Platynereis* eggs. However, I did not get any detectable signal, probably due to a below-threshold of the signal.

Therefore, I employed a transient transgene with even stronger SuperTOPFlash promoter with 8 Tcf/Lef binding sites and the gene for a more stable tdTomato fluorescent protein (courtesy of V. Kořínek) without an ambition of creating a transgenic line, although even non-integrating transgenes sometimes integrate in *Platynereis* (Z. Kozmik from P. Vopálenský, personal communication).

I injected this construct into the *Platynereis* zygotes and observed the fluorescence of tdTomato during the development (Figure 9B). At 24 hpf, I observed the highest tdTomato signal in the half of the larvae with lipid droplets that correspond to macromeres which later will give rise to the gut endoderm (see the section 7.3.7.3). After 1 week from the injection, tdTomato signal indeed marked the developing midgut and provides a further support for the hypothesis that the Wnt/β-catenin is active and could serve an important role in the developing midgut of *Platynereis* nectochaete larvae.

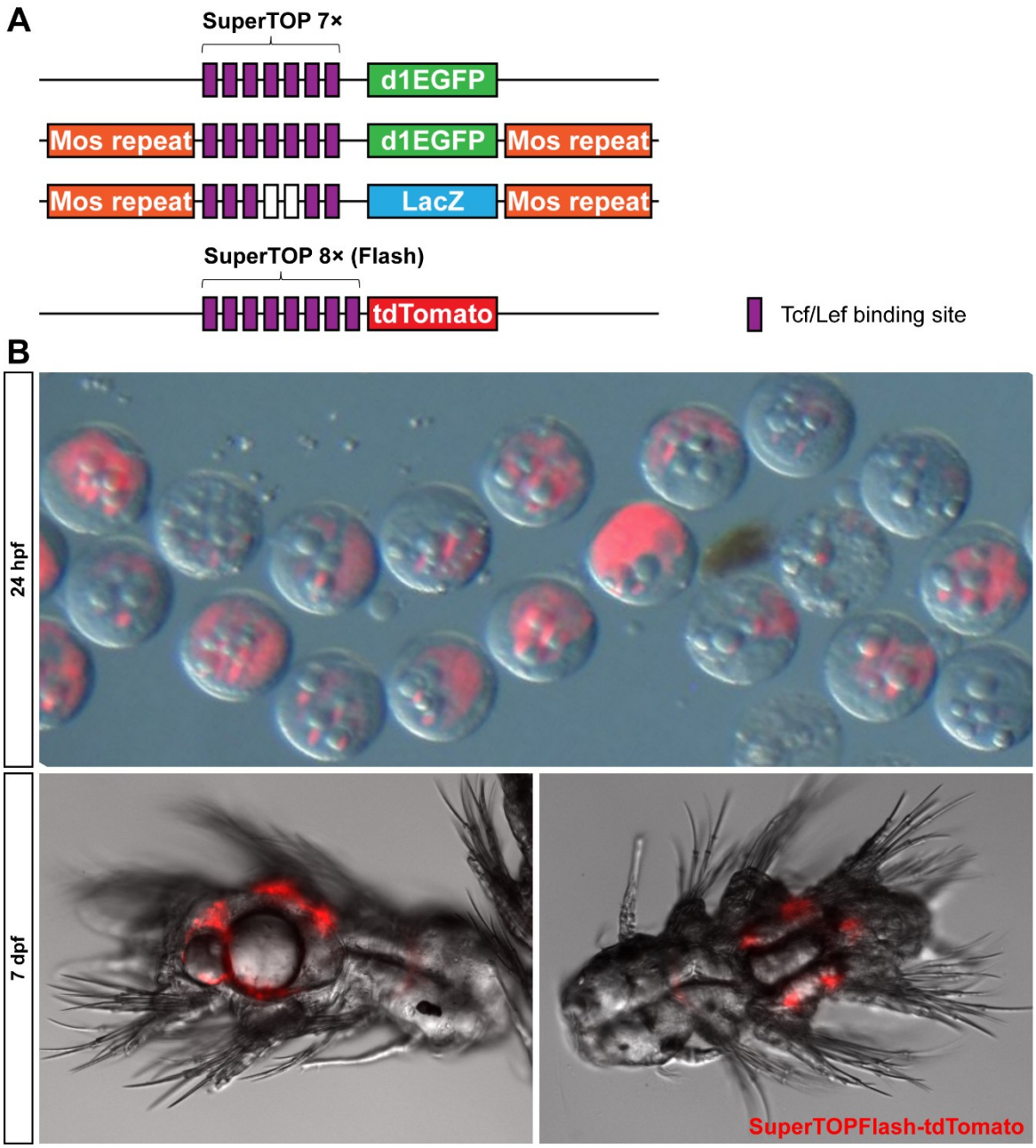


Figure 9 – Wnt/β-catenin responsive transgene is active in the gut

(A) Various Wnt reporter constructs created and injected into the *Platynereis* zygotes in the course of the project. From the top:
pSuperTOP-d1EGFP-N1 (courtesy of O. Machoň) is a transient non-integrating and pMos-SuperTOP-d1EGFP an integrating (when injected together with the mRNA for Mos transposase) transgene with destabilized EGFP under SuperTOP 7× promoter with 7 Tcf/Lef binding sites. pMos-SuperTOP-LacZ was made using the same integrating transgene, but carries LacZ instead of d1EGFP and lost two Tcf binding sites during cloning. I never observed any signal with these transgenic constructs. Therefore, I used a non-integrating transgenic construct SuperTOP (8×) Flash-tdTomato (courtesy of V. Kořínek), which provided bright fluorescence in transient transgenic larvae. *(the legend continues on the next page)*

(Figure 9 – continuation from the previous page)

(B) Red fluorescent signal was observed in the macromeres of 24 hpf larvae and in the endoderm of the 7 dpf transient transgenic larvae microinjected as zygotes with Wnt reporter construct Super-TOP (8×) Flash-tdTomato with 8 Tcf/Lef binding sites. The sites are bound by Tcf/Lef family of transcription factors and activate transcription only in the presence of stabilized β-catenin due to active canonical Wnt signalling. The presence of the signal in the gut indicates that the Wnt/β-catenin pathway is active here. Representative individuals are shown.

10.1.5. Tcf

In order to map better possible sites of action of the Wnt/β-catenin signalling in *Platynereis*, we set out to isolate a previously unknown homologue of the endpoint effector of the canonical Wnt signalling on the transcription of target genes, the transcription factor Tcf/Lef/Pangolin.

Dr. Ondřej Machoň, by a search in publicly available *Platynereis* EST database found two contigs that were homologous to mammalian Tcf. He used them to isolate two cDNA fragments of *Platynereis(Pdu)-Tcf*, one from the N-terminus and the other from the C-terminus of the Pdu-Tcf protein. I later used these primers to get a full length *Pdu-Tcf* cDNA as was confirmed by a protein alignment (Figure 10A) and phylogenetic analysis of the new sequence with other *Tcf/Lef/Pangolin* sequence from across the bilaterian phylogenetic tree (Figure 10B). However, the isolated *Pdu-Tcf* (despite it was terminated by a stop codon) did not possess a conserved C-terminal C-clamp domain which is present in all known Tcf proteins from organisms with a single *Tcf* homologue. Therefore, dr. Chrysoula Pantzartzi searched newly available *Platynereis* transcriptomic databases with the full length *Pdu-Tcf* cDNA and found two more *Pdu-Tcf* sequences that differed from our query sequence in their C-termini. Using these sequences, I was able to demonstrate they represent alternative products of a single *Pdu-Tcf* gene generated by an alternative splicing and isolate fragments of multiple *Pdu-Tcf* C-terminal isoforms (Figure 17). I used the cloned N- and C-terminal fragments to generate digoxigenin labelled RNA probes to detect *Pdu-Tcf* expression by *in situ* hybridization. The N-terminal fragment covered the sequence common to all *Pdu-Tcf* variants and gave a broader expression pattern than C-terminal probes that also encompassed the gut. I thus used the N-terminal probe for a thorough expression analysis in several developmental stages (Figure 11 – Figure 16).

10.1.5.1. Pdu-Tcf phylogenetic and sequence analysis

In order to confirm that the obtained sequence indeed belongs to a *Tcf* and to annotate its domain composition, I compared the longest possible Pdu-Tcf protein variant to Tcf/Lef/Pangolin protein sequences from representatives of major animal clades. The BLAST search with a translated Pdu-Tcf sequence returned exclusively Tcf sequences. In organisms with more *Tcf* genes in their genomes (vertebrates), the best BLAST hits belonged toTcf4 (Tcf7l2). This could suggest that not only the sequence but perhaps also the properties of Pdu-Tcf hence could be most similar to the vertebrate Tcf7l2, a vertebrate Tcf paralogue that confers either activating and repressive functions.

The phylogenetic analysis (Figure 10A) identified Pdu-Tcf with a high confidence as a closest relative to the previously published Tcf from another polychaete annelid *Perinereis nuntia* (Niwa et al., 2013) and both were placed within Spiralia.

The multiple sequence alignment of Tcf protein sequences (Figure 10B) revealed that Pdu-Tcf contains all major functional domains that are present in most known Tcf proteins (see the section 7.1.4.4). The N-terminus bears the activating β-catenin-binding domain, whereas the central part contains the inhibitory Groucho-binding sequence (GBS) (Arce et al., 2009) that binds Groucho co-repressor in the absence of Wnt signal (see the sections 7.1.4.1 and 7.1.4.4). The main HMG DNA-binding domain followed by a tail of basic amino acids is located close to the C-terminus and the accessory DNA-binding C-clamp domain on the very C-terminal end. Pdu-Tcf does not contain complete LPVQ/SXXSS motif which is present in vertebrate Tcf3 and repressive isoforms of Tcf7l2 (Liu et al., 2005). Although it does contain the second part of this motif (SXXSS), such sequence might be quite common and the lack of full signature suggests that Pdu-Tcf does not confer a repressive activity in the presence of Wnt signal.

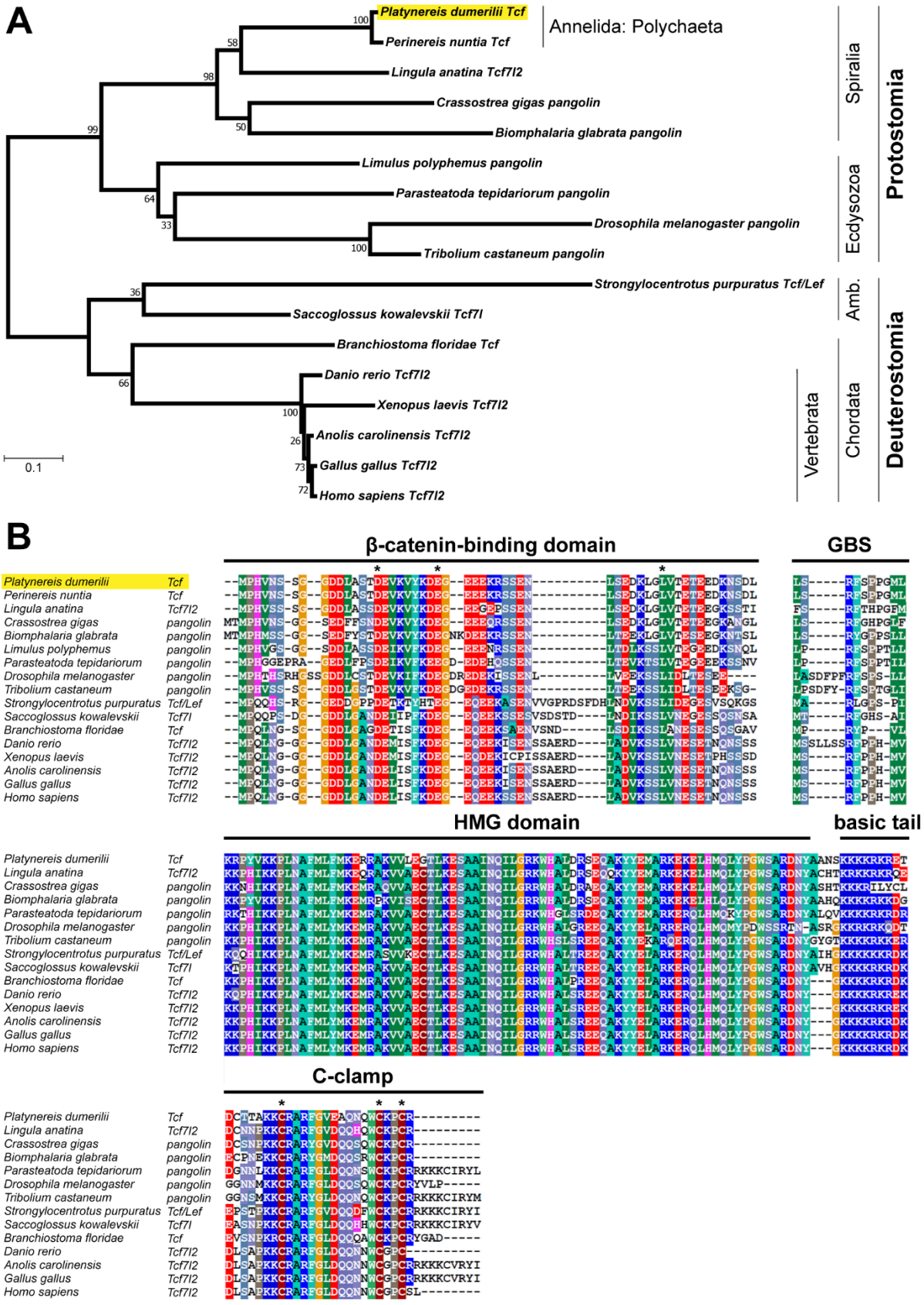


Figure 10 – Phylogenetic relationship and conserved domains of Pdu-Tcf

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Figure 10 – Phylogenetic relationship and conserved domains of *Pdu-Tcf*

(A) Phylogenetic tree of Pdu-Tcf protein (labelled in *yellow*) sequence with its top NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) hits from selected organisms. Pdu-Tcf clusters together with Tcf of another polychaete, *Perinereis nuntia*, to the spiralian lineage of bilaterian Tcf sequences and is most similar to Tcf7l2 from the groups where more *Tcf* genes are present. Protein sequences from taxa, where we did not detect a β-catenin binding domain using Pfam (Finn et al., 2016; Sonnhammer et al., 1997) in addition to an HMG DNA-binding domain and which disrupted the tree topology, were excluded from the analysis although their function in Wnt signalling was in some cases verified experimentally (e. g. *Caenorhabditis elegans* POP-1). The higher order taxa are indicated on the right; Amb. = Ambulacraria. The tree with the highest log likelihood (-6465.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

(B) Alignment of Pdu-Tcf with known Tcf protein sequences from other taxa reveals the conservation of core domains necessary for Tcf function – N-terminal β-catenin binding domain, GBS - Groucho binding sequence, HMG box DNA-binding domain with basic tail and the C-terminal C-clamp accessory DNA-binding domain, characterised by the presence of the CRARF/CRARY amino acid sequence. Asterisks mark conserved leucin and acidic amino acid residues within the β-catenin binding domain and cysteines in C-clamp domain. Sequences which were incomplete (*P. nuntia* and *L. polyphemus* for HMG domain) or lacked the domain (*Xenopus laevis* for C-clamp) were excluded from the alignment. The extent of highlighted domains corresponds to those published previously (Archbold et al., 2012; Cadigan and Waterman, 2012).

10.1.5.2. Expression of Pdu-Tcf in larval stages

I used a digoxigenin-labelled RNA probe antisense to the 5' region of the *Pdu-Tcf* mRNA which corresponds to the entire β-catenin binding domain to detect the expression pattern of *Pdu-Tcf* by fluorescent and standard *in situ* hybridization on whole mount *Platynereis* larvae of various stages.

In the early trochophore stage at 24 hpf (Figure 11 – first row), *Pdu-Tcf* is present at low levels without any distinct pattern in the ventral-lateral episphere in the ventral portion of the hyposphere in the neuroectoderm of the blastoporal lips.

One day later, at the onset of the metatrochophore stage at 48 hours of development (Figure 11 – second row; Figure 12 – first column from the left), *Pdu-Tcf* is strongly transcribed and displays a more distinct expression pattern in all germ layers. In the episphere, it is present in the region of both larval ocelli and the future adult eyes, in neurons

of brain ganglia and quite surprisingly also in the ciliated cells of the apical organ (marked by a red asterisk on the Figure 12). In the hyposphere. *Pdu-Tcf* can be found in two longitudinal domains in the neuroectoderm that from both sides abut the ventral midline. Laterally, it is expressed in the segmental stripes of the ectoderm, in the chaetal sacs and probably also the nearby forming mesoderm. *Pdu-Tcf* is excluded from most of the dorsal portion of the hyposphere and its expression extends from the dorsal border of the telotroch to the border between the ventral and dorsal halves of the prototroch.

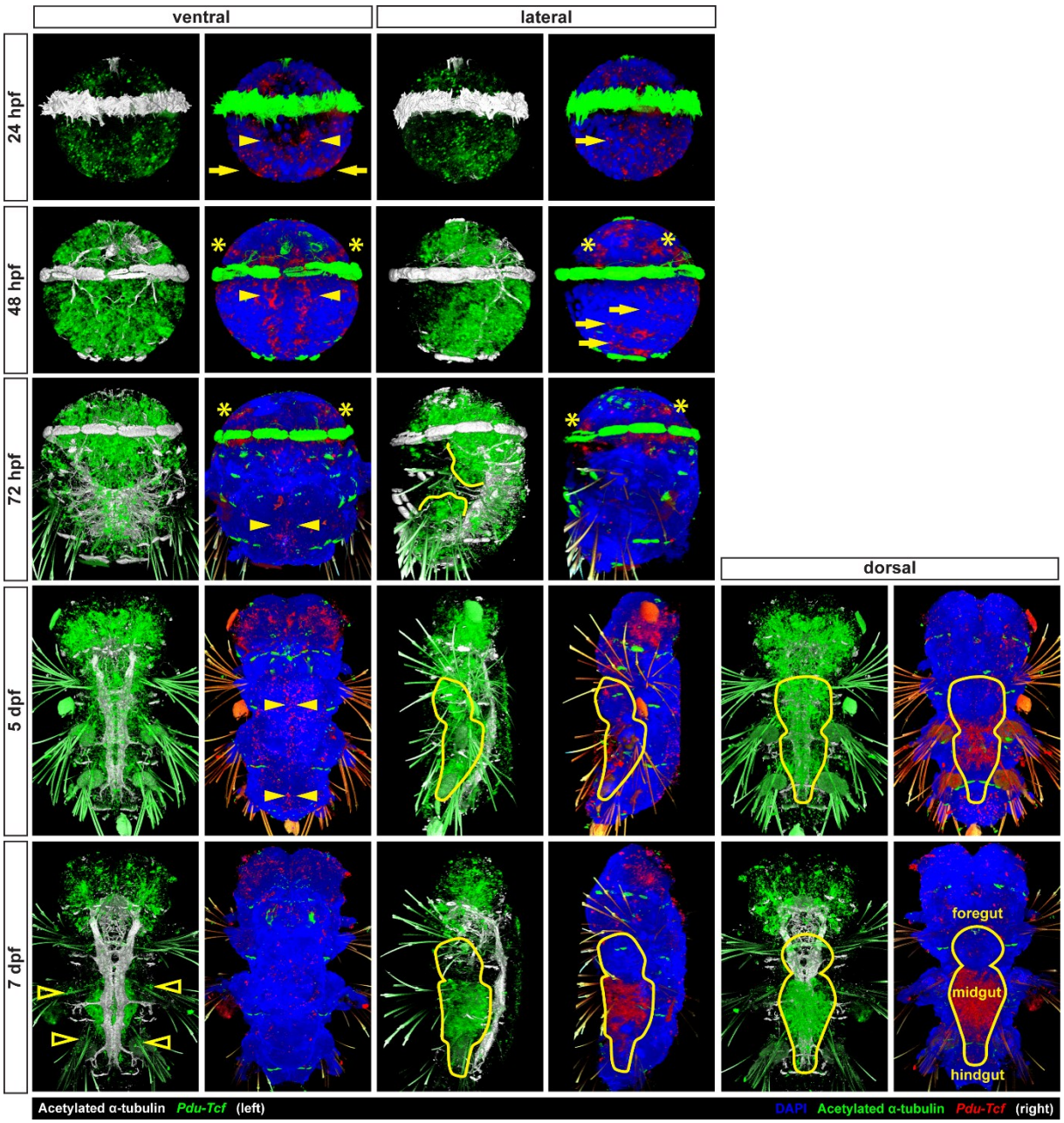

Figure 11 – Expression of *Pdu-Tcf* in various developmental stages of *Platynereis dumerilii*

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Figure 11 – Expression of *Pdu-Tcf* in various developmental stages of *Platynereis dumerilii*

In situ hybridization of *Pdu-Tcf* through the development of *Platynereis dumerilii* with the nuclei counter-stained with DAPI and the neuronal axonal scaffold and ciliary belts by immunofluorescent labelling of acetylated (stabilized) form of α-subunit of tubulin to highlight the overall morphology (*left: Tcf* green, acetylated tubulin white; *right – Tcf* red, DAPI blue, acetylated tubulin green). At 24 hpf, *Pdu-Tcf* is present broadly but at low levels in both the episphere and the hyposphere. At 48 hpf hyposphere, *Pdu-Tcf* is expressed in two parallel bundles of ectodermal cells that abut the ventral midline from both sides (*yellow arrowheads*). It is also present laterally and ventrally in the developing segments (*yellow arrows*). In the episphere, it can be found in the larval (ventral) and future adult (dorsal) eye regions (*yellow asterisks*). At 72 hpf stage, *Pdu-Tcf* expression becomes restricted mainly to the episphere and the stomodeal rosette, whereas it becomes scarcer in most of the hyposphere except the posterior developing proctodaeum. This trend continues throughout 5 dpf, where the strongest expression domains encompass the brain ganglia of the head lobes and the stomodaeum but can be observed to a lesser extent also in the rest of the developing gut. At 7 dpf, *Pdu-Tcf* is still expressed in the brain, however, a new strong expression is observed in the midgut and hindgut. There is also a small patch of *Pdu-Tcf* signal at the base of each parapodium (*empty arrowheads*). The expression patterns are described in greater detail in the text. The probe complementary to N-terminus of *Pdu-Tcf* mRNA, which should be the same for all C-terminal isoforms, was used and should thus detect all variants.
Approximate size of a 48 hpf larva after the hybridization procedure is around 130 μm, all images are to scale. Stage and orientation are indicated; anterior up; in lateral view ventral to the right. Representative individuals are shown.

By the end of the metatrochophore stage at 72 hpf (Figure 11 – third row; Figure 12 – second column from the left), the *Pdu-Tcf* expression persists in the larval and adult eye region, brain ganglia in the episphere. The two stripes of *Pdu-Tcf* expression along the ventral midline get closer to each other in the trunk, which now have become segmented. The segmental pattern of *Pdu-Tcf* in the lateral ectoderm is weak but still present. Strong *Pdu-Tcf* signal can be observed in the invaginating stomodaeum and in the posterior anal invagination that are migrating towards each other.

The mid-nectochaete stage at the day 5 of development (Figure 11 – third row; Figure 12 – second column from the right) witnesses the strongest *Pdu-Tcf* signal in the head neuroectoderm of cerebral ganglia (Figure 11), in isolated cells of the pharynx and in the hindgut. Only slight *Pdu-Tcf* expression can be observed in the midgut that has just begun to cellularize (transversal sections on the Figure 12). The expression in the trunk

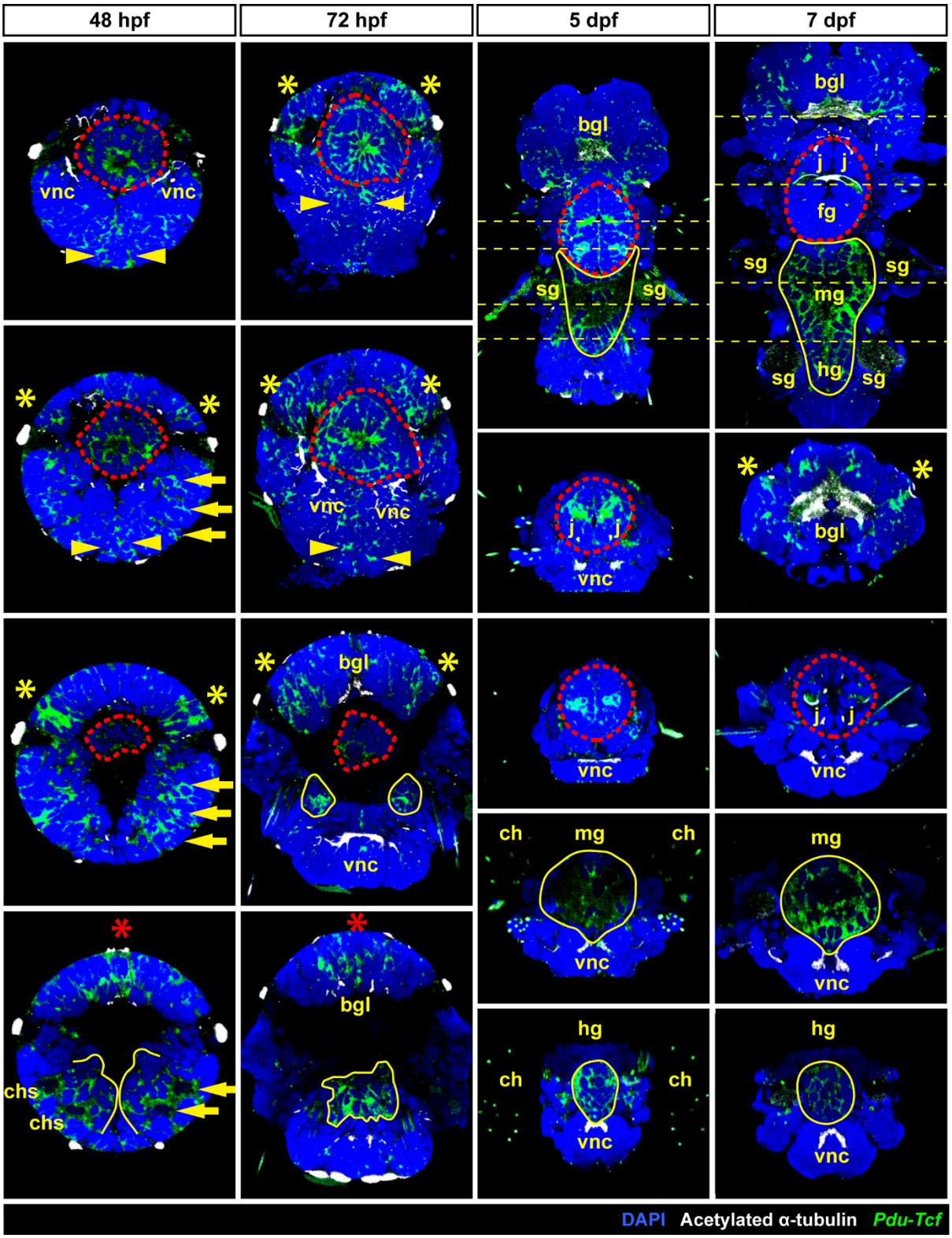

Figure 12 – Detailed analysis of *Pdu-Tcf* expression

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Figure 12 – Detailed analysis of *Pdu-Tcf* expression

To illustrate inner features and expression domains, virtual orthogonal sections through confocal fluorescent *z*-stacks of larvae after *in situ* hybridization with *Pdu-Tcf* N-terminal probe are shown. The nuclei were counterstained with DAPI (*blue*) and the axonal and ciliary scaffold was immuno-labelled with the antibody against acetylated form of α-tubulin (*grey*).

The stages are indicated; 48 and 72 hpf – coronal sections from ventral to dorsal (top-down); 5 and 7 dpf, top – coronal sections with the positions of transverse sections indicated by a *yellow dashed line*; below – consecutive transverse sections from anterior to posterior as indicated on the coronal sections.

red asterisk – apical organ, *red dashed line* – stomodeal rosette/pharynx (foregut), *yellow arrow* – segmental expression in the mesoderm of chaetal sacs, *yellow asterisk* – lateral larval eye regions in 48 and 72 hpf stage, putative adult eye region in 7 dpf larva, *yellow dashed line* – position of transverse sections, *yellow line* – a putative mesoderm at 48 hpf and the mesoderm and hindgut invagination at 72 hpf, midgut and hindgut at 5 dpf and 7 dpf.

bgl – brain ganglia, *ch* – chaetae, *chs* – chaetal sacs, *fg* – foregut, *hg* – hindgut, *j* – jaws, *mg* – midgut, *sg* – spinning glands, *vnc* – ventral nerve cord(s).

Approximate size of a 48 hpf larva is around 130 μm, other images (except for 7 dpf stage) are to scale. Representative individuals are shown.

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neuroectoderm is weaker and the two formerly paramedial (abutting the ventral midline) domains fuse into a single ventral-medial domain, which suggests that the former ventral midline has been internalized.

In the one week old (7 dpf) late nectochaete stage (Figure 11 – last row; Figure 12 – first column from the right; Figure 13), higher *Pdu-Tcf* is confined to the neuroectoderm of the developing sensory organs (antennae, palpae, cirri and the adult eyes) and appears in small patches at the bases of parapodia II and III. The expression in the pharynx (foregut) diminishes. A conspicuous new domain of strong *Pdu-Tcf* expression appears in the midgut, whereas it is only weakly expressed in the hindgut and can be revealed there only by prolonged staining (Figure 13A, B – left). This is consistent with the observed midgut occurrence of *Pdu-Axin* mRNA (chapter 10.1.2) and of the β-catenin protein (chapter 10.1.3). Another domain of *Pdu-Tcf* expression, again similarly to *Pdu-Axin*, is found in the ring of cells between the last trunk segment and the pygidium and probably represents the segment addition zone(SAZ), as Tcf was participates on segment addition in the SAZ of juvenile worms in the related species *Perinereis nuntia* (Niwa et al., 2013).

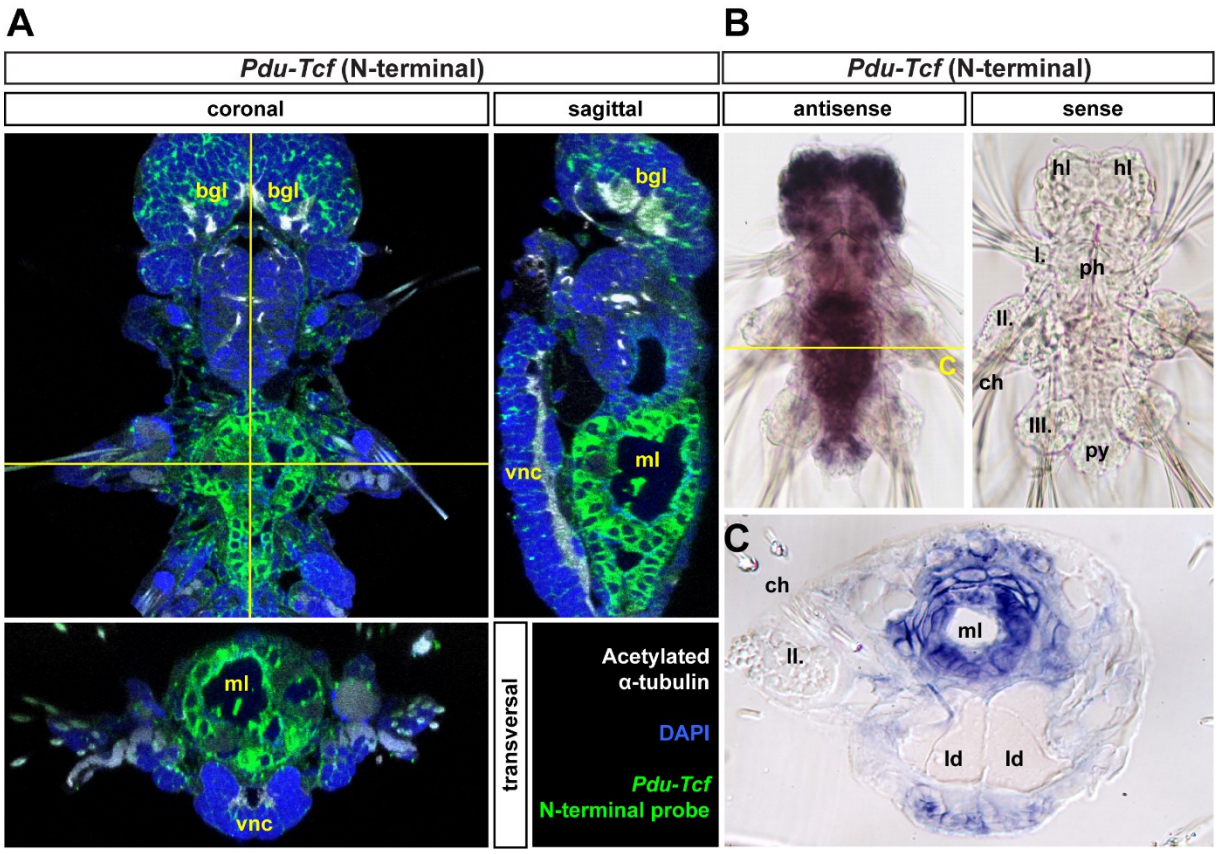

Figure 13 – Detection of *Pdu-Tcf* in the gut by N-terminal probe

(A) Virtual orthogonal sections through a confocal *z*-stack of a 7 dpf *Platynereis* larva after prolonged *in situ* hybridization staining with the N-terminal probe complementary to region that encodes β-catenin-binding domain show the expression of *Pdu-Tcf* in both the midgut and hindgut.
(B) *In situ* hybridization NBT/BCIP staining with antisense (left) and sense (right) probe against 5‘ end (corresponding to protein’s N-terminus) of *Pdu-Tcf*. The antisense probe (left) specifically detects *Pdu-Tcf* in the brain and in the gut whereas the sense probe (right) or hybridization without any probe (not shown) leads to an absence of signal showing that the observed signal is not due to unspecific binding of digoxigenin-labelled probe. Moreover, the latter also demonstrate that the observed signal yielded with the antisense probe was not due to persisting endogenous alkaline phosphatase activity (which was thus successfully inactivated by hybridization temperature). This is further supported by a fact, that a treatment with levamisole (an inhibitor of alkaline phosphatase) did not abolish the signal seen with antisense probe (not shown).
(C) Physical thin transversal section through the body of the 7 dpf larva after *in situ* hybridization with the 5‘-end (N-terminal) *Pdu-Tcf* mRNA antisense probe confirms its presence in the gut. The section is taken approximately on the level of the second segment, i. e. through the midgut.
I., II., III. – parapodia of the first, second and third body segment, *bgl* – brain ganglia, *hl* – head lobes, *ld* – lipid droplets, *ml* – midgut lumen, *ph* – pharynx (foregut), *py* – pygidium, *vnc* – ventral nerve cord. Representative individuals are shown.

To verify that the staining in the gut is specific and it is not a result of the activity of endogenous alkaline phosphatase (AP), which naturally occurs in the gut (Hasse et al., 2010), I repeated the experiment with larvae that were treated with the AP inhibitor levamisole prior to staining. The levamisole treatment did not abolish the staining (not shown) which also worked with another AP substrate (Figure 13 – left). The same procedure with *Pdu-Tcf* sense probe produced no staining and excluded a probe trapping as a possible source of the gut signal (Figure 13B – right).

10.1.5.3. The feedback regulation of Pdu-Tcf

It turned out that pharmacological treatments of developing *Platynereis* larvae with activator or inhibitor of Wnt/β-catenin pathway affected also the expression of *Pdu-Tcf* (Figure 14) which suggests a regulation by feedback loop(s). From 24 to 72 hpf, either activation or inhibition cause a drop in the levels of *Pdu-Tcf* mRNA. This points out to the existence of both positive and negative feedback loops or a misregulation of cell fate upon either activation or inhibition of the Wnt/β-catenin pathway.

Conversely, at 7 dpf the activation of Wnt/ β-catenin signalling by CHIR99021 results in the expression of *Pdu-Tcf* comparable but slightly higher than in controls, especially in the brain and the pygidial domain. After the inhibition by IWR-1-endo, somewhat lower level of *Pdu-Tcf* transcripts in the gut is accompanied by their higher amount in the head and diffuse low ectopic expression in the rest of the larval body. This could suggest a dual type of regulation – a positive feedback loop in the gut vs. a negative feedback loop in the rest of the body. This could correspond to different requirements for Wnt signalling in these tissues – high activity in the gut and precisely regulated one in the nervous system.

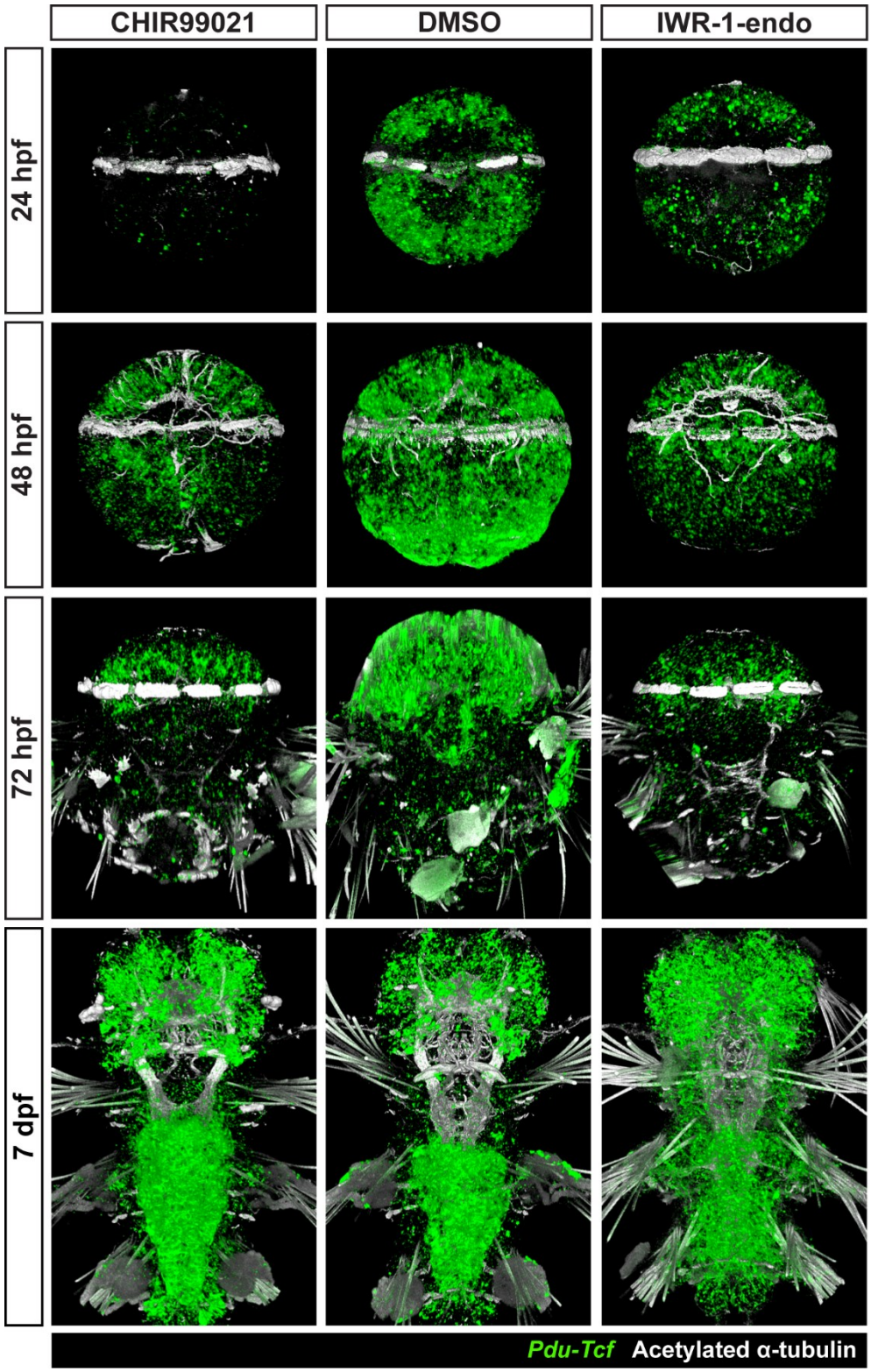

Figure 14 – The effect of pharmacological manipulation of Wnt/β-catenin pathway activity on *Pdu-Tcf* expression

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Figure 14 – The effect of pharmacological manipulation of Wnt/β-catenin pathway activity on *Pdu-Tcf* expression

3D projections of confocal fluorescent *z*-stacks show the expression of *Pdu-Tcf* as detected by *in situ* hybridization with N-terminal probe in controls (DMSO) and after activation (CHIR99021) or inhibition (IWR-1-endo) of Wnt/β-catenin signalling pathway on various *Platynereis* developmental stages.
At 24, 48 and 72 hpf stages, both activation and inhibition of canonical Wnt signalling lead to lower levels of *Pdu-Tcf*, most notably to the loss of terminal expression in 48 hpf larvae, suggesting a simultaneous existence of both positive and negative regulations and a need for balanced signalling for proper expression at these stages.
At 7 dpf, activation of Wnt/ β-catenin signalling results in the expression of *Pdu-Tcf* comparable to controls with slightly higher expression in the brain and pronounced expression domain in the pygidium, probably the segment addition zone. After inhibition, a somewhat lower level of *Pdu-Tcf* is observed.
All images are ventral views with anterior to the top. Approximate size of a 48 hpf larva is around 130 μm, all images are to scale. Representative individuals are shown.

10.1.5.4. Expression of Pdu-Tcf in the juvenile worm

Eventually, I investigated, whether *Pdu-Tcf* continues to be expressed in the amputated tails (that are able to regenerate and the worms survive) and in the head of growing juvenile atokous worms.

In the head and anterior segments (Figure 15), *Pdu-Tcf* was observed in the sensory organs, e. g. the palpae, the circumoesophageal connectives, the ventral nerve cord and in parapodia at the basis of chaetae both almost none was detected in the part of the gut immediately behind the jaws.

In the posterior part of the body (Figure 16), a strong *Pdu-Tcf* signal is found on the luminal surface of the gut in the digestive epithelium whereas the most β-catenin is detected closer to the basement membrane (Figure 16B, C, E, H). The gut runs through the entire body with regularly spaced constrictions which, however, do not coincide exactly with the septa between each segment (Figure 16B, C, G, H). A strong *Pdu-Tcf* signal is observed in bilateral groups of cells located in the body cavity in the space on both sides of these constrictions between individual gut compartments (Figure 16H). These groups of *Pdu-Tcf* positive cells located might represent the gonial clusters of germ cell progenitors.

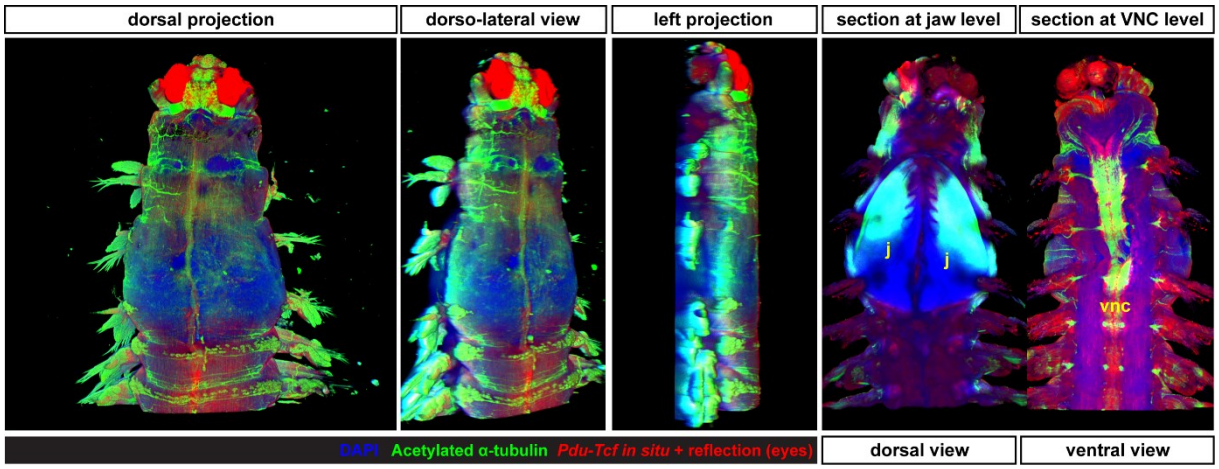

Figure 15 – Expression of *Pdu-Tcf* in the head of the juvenile *P. dumerilii*

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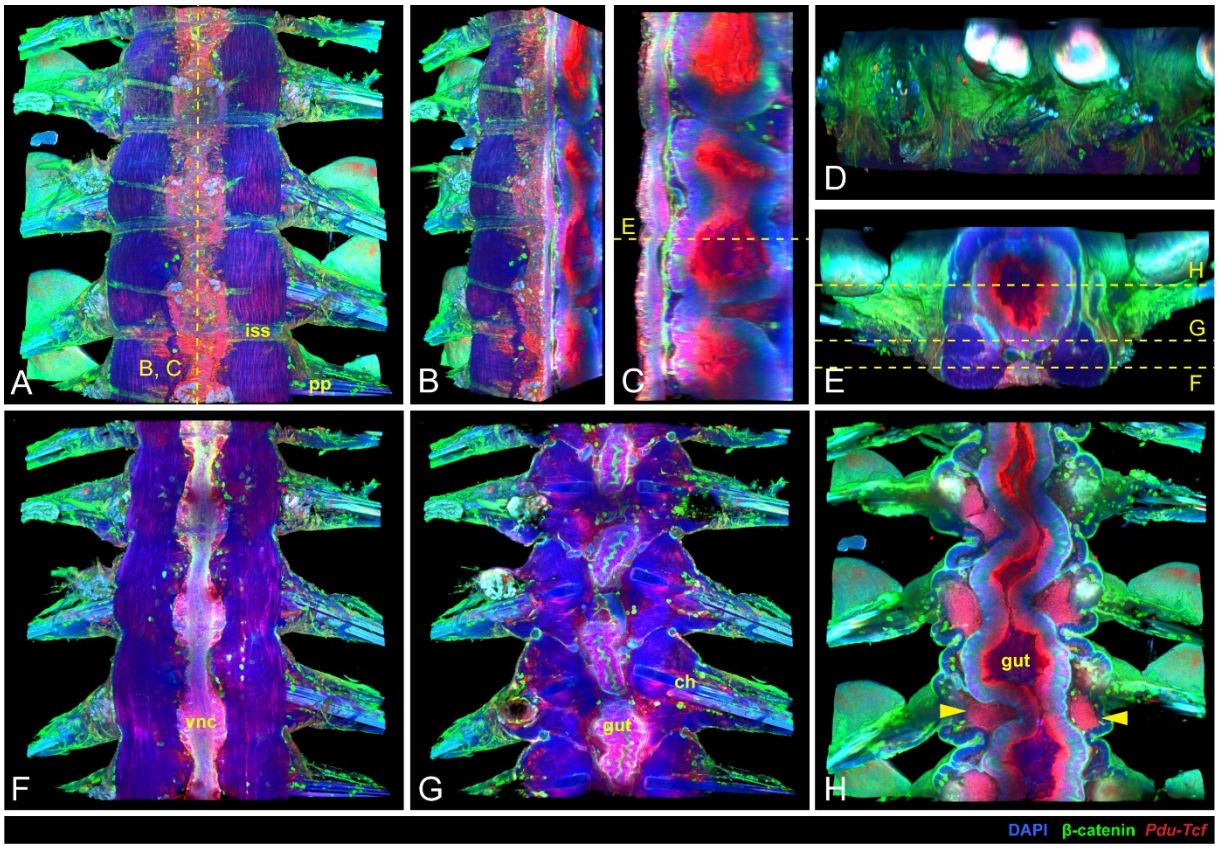

Figure 16 – Expression of *Pdu-Tcf* in the trunk segments of the juvenile *P. dumerilii*

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Figure 15 – Expression of *Pdu-Tcf* in the head of the juvenile *P. dumerilii*

3D projections of a confocal fluorescent *z*-stack of *Pdu-Tcf* N-terminal probe *in situ* hybridization on head and few first segments of an adult atokous worm. The specimen has been stained by DAPI and immunolabelled by the antibody against acetylated α-tubulin to visualize the morphology. The eyes were imaged by detecting the light from 635 nm laser reflected by eye pigment. *Pdu-Tcf* can be seen in the frontal-ventral head (more precise description is prevented because of shading by the eye pigment), in the ventral nerve cord (*vnc*), to a lesser extent in muscle bundles parallel to it and in parapodia.
j – jaws, *vnc* – ventral nerve cord. Anterior is up. Representative individual is shown.

Figure 16 – Expression of *Pdu-Tcf* in the trunk segments of the juvenile *P. dumerilii*

(A) 3D reconstruction of a confocal *z*-stack of several trunk to tail segments of adult epitokous *Platynereis* showing the expression of *Pdu-Tcf* by fluorescent *in situ* hybridization (*red*), counter-stained with DAPI (*blue*) and β-catenin immunolabelling (*green*) to visualize cell nuclei and the body surface; ventral view, anterior up. Representative individual is shown.
(B) 3D projection of sagittally sectioned half of the *z*-stack from (A). The section was taken through the midline. Ventrolateral view, anterior is up.
(C) The same section in lateral view, ventral to the left, anterior up. Strong *Pdu-Tcf* expression signal in the intestine and constrictions of the gut between segments are apparent.
(D) Lateral view of the whole *z*-stack from (A); ventral down, anterior to the left.
(E) Transverse section of the same *z*-stack through the middle of the segment indicated in (C) with the planes of coronal sections in (F), (G) and (H) indicated. Ventral is down. Gut cavity and the surrounding gut epithelium with strong *Pdu-Tcf* signal are apparent. On the ventral side, ventral nerve cords display relatively high *in situ* signal compared to the neighbouring ventral longitudinal muscles.
(F) Coronal section through the *z*-stack from (A) on the level of the ventral nerve cord (vnc) as marked in (E). Some *in situ* signal is present in the vnc and weakly in the ventral longitudinal muscles.
(G) Another coronal section taken deeper in the body through the intestinal wall. The expression of *Pdu-Tcf* can be seen in the gut and in few cells around the base of chaetae. β-catenin staining is strongest on the surface of the body and on the wall of the gut.
(H) Coronal section through the middle of the gut demonstrates strong *Pdu-Tcf* intestinal expression, especially on the luminal side, and in the intersegmental clusters of mesodermal cells (marked by *yellow arrowheads*), which are placed in every constriction of the gut and could represent the gonial clusters and/or the excretory system. Anterior is up for (F), (G) and (H).
ch – chaetae, *iss* – intersegmental septum, *pp* – parapodia, *vnc* – ventral nerve cord.

There is a *Pdu-Tcf*-positive thickening of/around the VNC in every segment and a lower amount can be seen in the ventral longitudinal muscles (Figure 16A, F). If we take in to account that Wnt signalling often maintains tissue specific stem cells and thus marks the sites of cell proliferation and tissue growth, it raises the possibility that the segmental *Pdu-Tcf*-positive thickenings in the vicinity of the VNC in the growing juvenile worms are the sources of the cell proliferation and the sites of production of the new tissue for the growth of the segments in their width, i. e. the widening of the segment. This is necessary as the juvenile worm not only add new segments and grows in length, but also has to thicken in order to maintain more or less constant proportions of the body.

Spots of *Pdu-Tcf* expression at the base of chaetae suggest a possible role of Wnt signalling in their growth, similar to the maintenance of hair follicle stem cells in mammals.

10.1.5.5. Pdu-Tcf isoforms

I set out to find out whether the *Pdu-Tcf* sequences from the available transcriptomes, that differed in their C-termini, represent different genes or just splice variants of a single gene.

To achieve this goal, I used forward primers that were specific to the variable region situated upstream to the HMG domain and a common reverse primer with the sequence of the very end of the longest *Pdu-Tcf* mRNA (Figure 17A) and used them with mixed stage cDNA as a template. I obtained three fragments of different sizes from each combination (Figure 17B), which suggested that these variants are generated by an alternative splicing.

To confirm this hypothesis, I used a forward primer specific to the end of HMG DBD coding region and the same reverse primer with *Platynereis* genomic DNA template. A comparison with cDNA sequences revealed that the cloned genomic fragment contained an exon which encodes the C-clamp domain of Pdu-Tcf with cryptic three alternative splice sites. They allow to generate *Pdu-Tcf* mRNA variants with C-clamp domains of three different lengths, but all containing the CRARY aa signature, from a single gene. Moreover, if the splicing does not take place at all, a cryptic termination codon within the intron just after the HMG domain exon cause a premature termination of translation and generate a splice protein variant that omits the C-terminus with C-clamp domain entirely. All these three alternative C-clamp (+) and one C-clamp (–) variants can freely combine with putative alternative exons which encompasses the region upstream of HMG and the beginning of HMG. Therefore, they can give rise to as many as 8 different Pdu-Tcf C-terminal variants

(Figure 17C), which I am calling here provisionally "Pdu-Tcf isoforms X1 to X8" (X means that they are not assigned to the functional and structural groups used in vertebrate Tcf proteins). Note that they do not necessarily represent actual isoforms as there might be more alternative exons coding for the N-terminal and central part of the Pdu-Tcf protein.

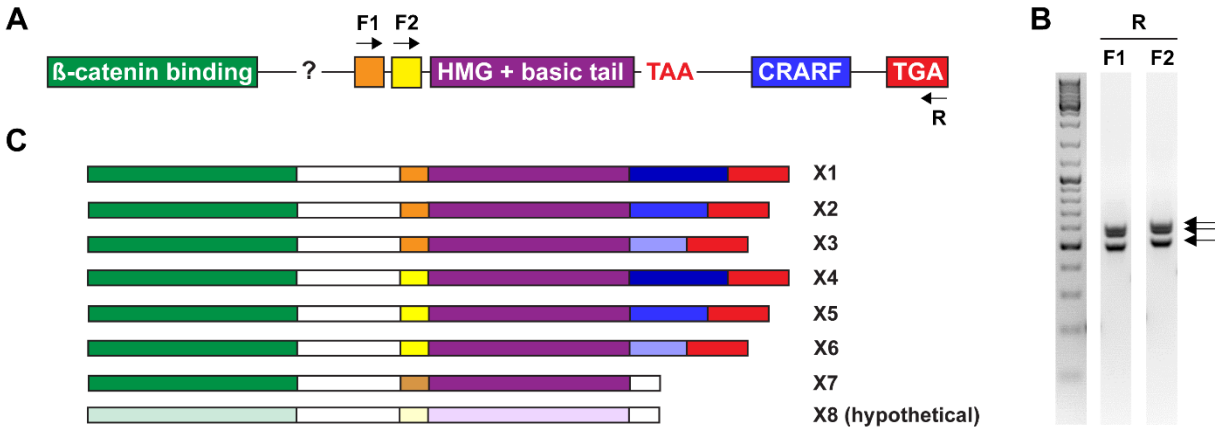


Figure 17 – A single Platynereis Tcf gene produces multiple isoforms

(A) Exon-intron structure of *Pdu-Tcf* C-terminus as described in this work; N-terminal β-catenin binding domain is composed of at least 3 exons which is not depicted here; two alternative exons exist for the beginning of HMG DNA-binding domain, followed by an exon common for both variants of the HMG domain. A premature stop codon within the intron that follows the HMG exon, if not excised by splicing, causes a premature termination of translation and produces C-terminally truncated Tcf ending immediately after the HMG domain. There is only a single C-clamp (CRARF) exon but it contains 3 alternative splice sites to produce 3 C-clamp variants in combination with a common C-terminal exon. Position of primers used to isolate the individual *Pdu-Tcf* isoforms is indicated.
(B) The result of PCR on the mixed-stage cDNA template with the primers indicated in (A). Arrows point at three bands corresponding to three different splice variants amplified in each PCR reaction.
(C) Graphic representation and classification of identified isoforms of *Pdu-Tcf* regarding to its C-terminus. I was not able to amplify the isoform X8, which thus remains theoretical, and I detected the isoform X6 only as a PCR product on a gel [compare to (B)] but the attempts to isolate it, clone it and sequence were not successful. Note that these isoforms are defined only according to their C-termini and do not show any potential diversity of exons and alternative splicing in the N-terminal β-catenin binding domain or in the middle region between this domain and C-terminal exons. There is a small region in the β-catenin binding domain which can facultatively be not incorporated into the final product (not depicted here). Thus, each of so called "isoforms" which are listed here can actually split into two or more real isoforms.

In fact, there is a 36-nucletide sequence that corresponds to 12 amino acids and which is only facultatively included into the *Pdu-Tcf* mRNA and the resulting protein (see also the section 9.3.3). This sequence seems to be annelid- or polychaete-specific as the same sequence can be found in the Tcf of another polychaete *Perinereis nuntia* (Niwa et al., 2013). However, it is not present in the Tcf of any other organisms which I have examined. I thus used the N-terminal *Pdu-Tcf* antisense probe that did not contain this sequence for the detection of *Pdu-Tcf* expression by *in situ* hybridization but I included it into the *Pdu-Tcf* protein sequence used for phylogenetic analysis. This facultative region is located downstream of the second intron in the 3' end of the *Pdu-Tcf* gene, within the coding region of β-catenin binding domain. I do not know whether this sequence is coded by a separate alternative exon or is generated from an alternative splice site of a longer exon. As was already mentioned in the chapter 7.3.1, the genome of *Platynereis* is in its characteristics more similar to vertebrates than to compact genomes of other protostome models organisms and contains large genes with numerous introns (Raible et al., 2005). To dissect the complete exon-intron composition of *Pdu-Tcf* between the second intron and the HMG would require to design several new primers randomly distributed in this region and assemble the entire *Pdu-Tcf* gene from smaller fragments which did not seem necessary at the moment.

The diversity generated by alternative splicing affects the functional domains and can compensate for the lack of gene diversity and generate *Pdu-Tcf* functional variants comparable to multiple *Pdu-Wnt* and *Pdu-Fz* genes or four vertebrate orthologues (which themselves produce multiple isoforms). There was an assumption that Pdu-Tcf isoforms might have overlapping, but distinct expression patterns and functions and the expression pattern revealed by the N-terminal probe that could not discriminate among these isoforms is a composite from the expression patterns of individual isoforms. I was thus curious what functional domains contains Pdu-Tcf isoform expressed in the gut. For this purpose, I performed *in situ* hybridization stainings with isoform-specific C-terminal antisense probes of 6 out of 8 presumed C-terminal *Pdu-Tcf* variants on wild type *Platynereis* larvae fixed on the day 7 of development (Figure 18). Surprisingly enough, all these similar but yet different probes gave almost identical expression patterns but none of them displayed the expression in the gut revealed by the N-terminal probe. Instead, they showed an expression of these *Pdu-Tcf* isoforms in the cerebral ganglia and small patches at the bases of the second and third parapodia, probably the ventral segmental ganglia.

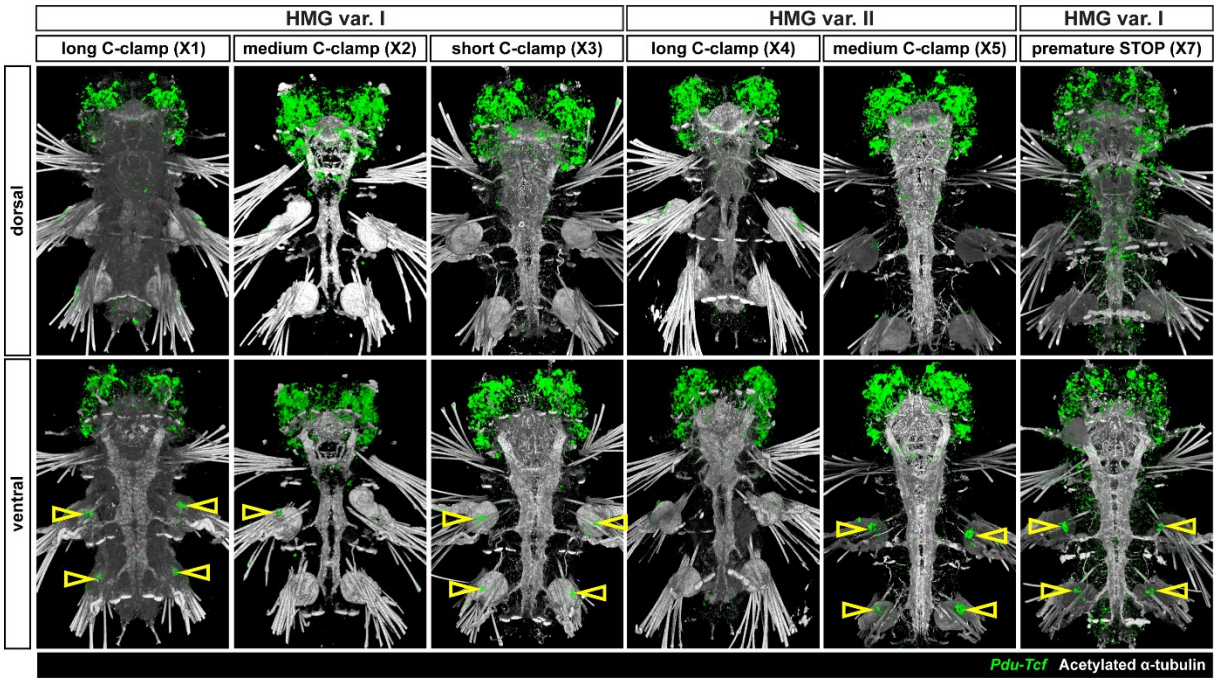


Figure 18 – Various *Pdu-Tcf* isoforms display the same expression pattern

Fluorescent *in situ* hybridization with probes specific to individual C-terminal isoforms X1 to X5 and X7. Expression pattern for all isoforms is essentially the same, i. e. they are all present mostly in brain ganglia. Weak signal can also be observed in small patches of few cells at the base of parapodia (*empty arrowheads*) and a weak signal is present in some cases [most prominent for C-clamp (-) isoform] in the pygidium. None of the tested isoforms shows any strong expression in the gut similar to 5‘ (N-terminal) probe against the region encompassing β-catenin binding domain, which should be common to and detect all isoforms.

All images are ventral views of 3D projections with anterior to the top. Representative individuals are shown.

10.2. Neuroectoderm development

10.2.1. Neurogenesis

Neurogenin drives the expression of pro-neural or neurogenesis-related genes (Seo et al., 2007), hence it marks undifferentiated neuronal cells and is implicated in their differentiation. Mammalian *Neurogenin* is a direct target of the canonical Wnt/β-catenin signalling (Hirabayashi et al., 2004) and is upregulated by Wnt/β-catenin in the dorsal cerebral cortex (Backman et al., 2005).

To find out more about the neurogenesis in *Platynereis* even before a detailed study was published (Demilly et al., 2013), I performed *in situ* hybridization with a *Platynereis* homologue of mammalian *Neurogenin* closest related to the sequence of *Neurogenin2*, hence *Pdu-Ngn2*. In control 48 hpf larvae treated with DMSO, it was expressed in the entire ventral neuroectoderm of the hyposphere and almost complete ectoderm of the episphere (Figure 19 – middle). Consistent with the observations in vertebrates, the inhibition of Wnt/β-catenin signalling by IWR-1-endo caused a severe reduction in the *Pdu-Ngn2* (Figure 19 – right). Unexpectedly, the activation of Wnt/β-catenin signalling led to almost the same(Figure 19 – left).

I constantly observed a reduced number (zero or one instead of usual three) of the commissures between the ventral nerve cords in all treatments done in the time window from 24 to 48 hpf. Again, this effect was similar when either the activator or inhibitor of Wnt/β-catenin signalling pathway was used.

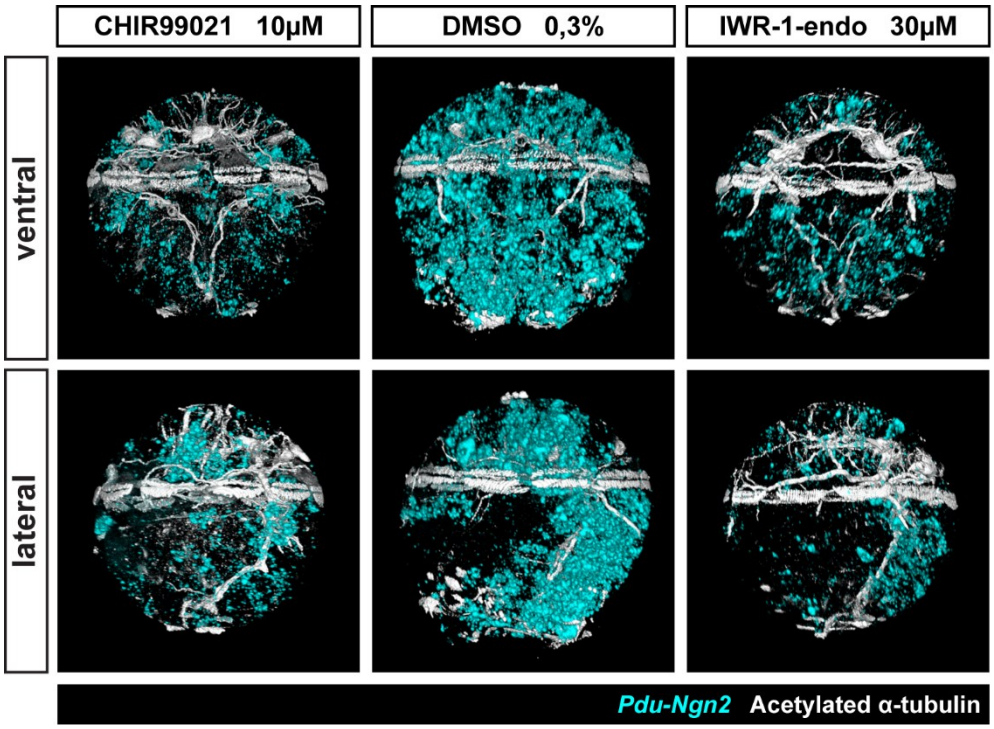


Figure 19 – Balanced Wnt/β-catenin signalling is necessary for neurogenesis

Both activation of Wnt/β-catenin pathway by CHIR99021 or inhibition by IWR-1-endo from 24 to 48 hpf have strikingly similar effect on the expression of *Pdu-Ngn2* (the *Platynereis* homologue of *Neurogenin 2*) gene (*cyan*) in 48 hpf larvae. There is a lot less *Ngn2* and thus neurogenesis when the pathway is ubiquitously activated or inhibited, which suggest that balanced or localized activity of Wnt/β-catenin pathway is required for proper neurogenesis. Note also the lower number of commissures between the two ventral nerve cords.

The images are 3D reconstructions of confocal *z*-stacks of *Pdu-Ngn2* fluorescent *in situ* hybridization; anterior is up, in lateral views ventral to the right. Approximate size of a 48 hpf larva is 130 µm. Representative individuals are shown.

10.2.2. Medio-lateral patterning

The lateral to medial sequence of transcription factors expressed in the neuroectoderm of protostomes from either Ecdysozoa (*Drosophila*) or Spiralia (*Platynereis*) corresponds to that of the dorso-ventral (D-V) patterning system in the vertebrate neural tube (Arendt et al., 2008). However, Wnt/β-catenin signalling has been implied to participate on the D-V patterning in the vertebrate neural tube but not in the neuroectoderm of *Drosophila* (cf. the section 7.2.3.2), To determine the ancestral state and the role of Wnt signalling in the nervous system development not only in *Platynereis*, it is thus crucial to answer whether Wnt/β-catenin signalling is involved in the medio-lateral patterning of neuroectoderm in Spiralia, of which *Platynereis* is a representative member.

10.2.2.1. Pax genes

Of the medio-lateral patterning genes, I selected the class of *Pax* genes. *Nk* genes have complementary expression patterns to *Pax* genes and it can be presumed that if there are any shifts in the boundaries of gene expression, they will be revealed already by *Pax* genes alone. I used antisense RNA probes to reveal the expression of *Pdu-Pax6*, *Pdu-Pax3/7* and *Pdu-Pax2/5/8* by *in situ* hybridization after the pharmacological manipulation of Wnt/β-catenin pathways from 24 to 48 hpf and for *Pdu-Pax6* also from 48 to 72 hpf (Figure 20).

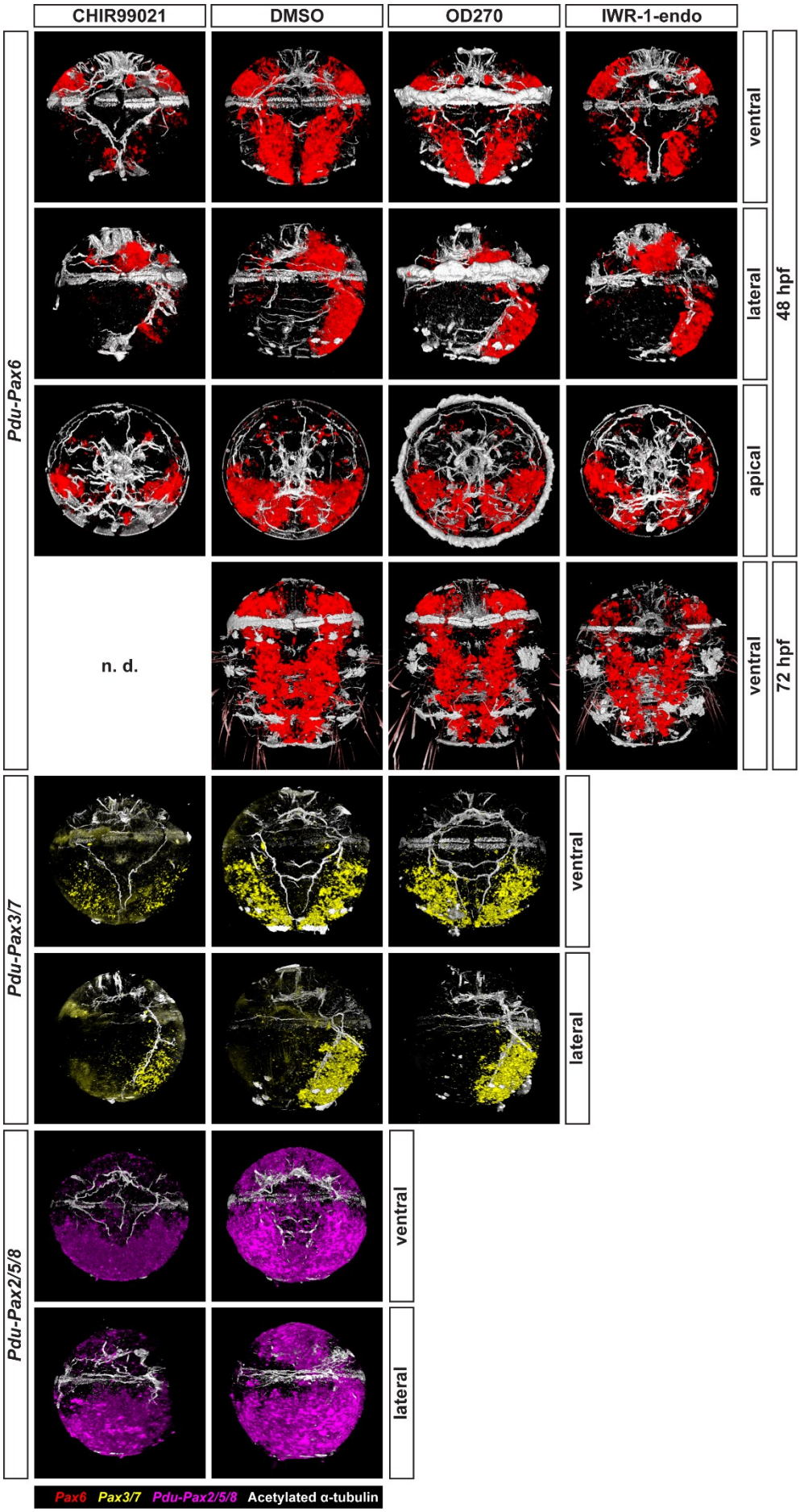
At 48 hpf, *Pdu-Pax6* is expressed most medially from the three *Pdu-Pax* genes in two longitudinal domains of the hyposphere neuroectoderm over the ventral nerve cords. The domains of *Pdu-Pax6* expression in the trunk follow the circumoesophageal connectives, diverge to circumvent the stomodaeum and continue as two domains on lateral sides of the episphere. It is also present in few cells of the dorsal brain ganglia (apical view of DMSO control treatment on the Figure 20) that might correspond to interneurons of future adult eyes (Z. Kozmik and R. Žídek, unpublished). The expression domains from 48 hpf persist to 72 hpf, when *Pdu-Pax*6 can be found also in the neurons of the commissures between the VNCs. The most striking phenotype of *Pdu-Pax6* expression was observed after the activation of Wnt/β-catenin signalling by CHIR99021. *Pdu-Pax6* mRNA almost diminishes from most of the ventral trunk neuroectoderm at 48 hpf except the posterior end of VNCs, where a faint *Pdu-Pax6* expression still can be observed. The expression domains in the episphere seem to be preserved but somewhat reduced in size.

The inhibition of Wnt/β-catenin by a weak inhibitor OD270 causes a narrowing of the expression domains in the ventral neuroectoderm and consequently a widening of the gap that separates them. *Pdu-Pax6* expression in the episphere retreats to the prototroch, i. e. it diminishes from the area closer to the apical organ. However, no such effect was observed with the strong Wnt inhibitor IWR-1-endo and might thus represent just a variability in staining of the examined OD270 individual. The overall expression after the Wnt inhibition by IWR-1-endo is similar in intensity to the DMSO control, but the *Pdu-Pax6* domains are reduced in size and *Pdu-Pax6* may be entirely absent from the putative dorsal cells of adult eyes in the episphere; nevertheless, to detect reliably the expression or absence of *Pdu-Pax6* in these cells requires a prolonged staining, as the expression there is even under normal circumstances low. I did not observe any pronounced effect on *Pdu-Pax6* expression after the inhibition of Wnt/β-catenin signalling by either OD270 or IWR-1-endo between 48 and 72 hpf. Therefore, I conclude that the specification and patterning of the neuroectoderm is already largely finished by the beginning of the metatrochophore stage at 48 hpf.

Pdu-Pax3/7 is confined solely to the ventral neuroectoderm where it is expressed more laterally but overlapping with *Pdu-Pax6* in longitudinal domains that send out segmental stripes on their outer edges. Unlike the other two *Pdu-Pax* genes, I did not observe any *Pdu-Pax3/7* expression in the episphere. The pharmacological treatment by the Wnt/β-catenin activator CHIR99021 strongly reduced the expression of *Pdu-Pax3/7*, whereas upon the weak inhibition by OD270, the domain of *Pdu-Pax3/7* was slightly broader and its medial border shifted a bit closer to the ventral midline, but this could be explained by a deformation of the sample.

Pdu-Pax2/5/8 displays the widest expression in the ectoderm and reaches most dorsally beyond the ventral neuroectoderm in the hyposphere as well as in the episphere. However, the whole-body activation of Wnt/β-catenin signalling leads to a severe reduction of *Pdu-Pax2/5/8* expression in the episphere, it diminishes from the ventral neuroectoderm and persists only in the dorsal and lateral trunk ectoderm.

I mention the expression of the ventral/medial marker *Pdu-Nk2.1* in the next chapter that is dedicated to the anterior-posterior patterning, as it is expressed mostly in the episphere (section 10.2.3.1) and the TF *Pdu-Emx* due to its pronounced segmental expression in the chapter that concerns the segmentation (chapter 10.3), although is better known as a marker of the vertebrate dorsal telencephalon.


CHIR99021
DMSO
OD270
IWR-1-endo
Pdu-Pax6
ventral
lateral
apical
48 hpf
n. d.
ventral
72 hpf
Pdu-Pax3/7
ventral
lateral
Pdu-Pax2/5/8
ventral
lateral
Pax6 Pax3/7 Pdu-Pax2/5/8 Acetylated α-tubulin

(from the previous page)

Figure 20 – Medio-lateral sequence of *Pax* genes

The images show expression patterns of three *Platynereis Pax* genes at 48 hpf and for *Pdu-Pax6* also at 72 hpf in normal conditions (DMSO control) and upon pharmacological modulation of Wnt/β-catenin signalling pathway. The stages for *Pdu-Pax6*, experimental condition and orientation are indicated.

In the presence of DMSO only, *Pax* genes are expressed in a medio-lateral sequence of overlapping domains with *Pdu-Pax6* being most medial, followed by *Pax3/7* and *Pax2/5/8* most lateral. *Pdu-Pax6* is expressed in the cells of ventral nerve cords (in the ectoderm above their axonal tract) in two longitudinal stripes abutting the ventral midline in the hyposphere. *Pax3/7* does not reach the ventral nerve cords but extends more laterally in segmental pattern. *Pax2/5/8* is broadly expressed in the lateral neuroectoderm. *Pdu-Pax6* is also present in two ventrolateral domains (the larval eye region) in the episphere and weakly in few dorsal cells of the future adult eyes. For *Pdu-Pax6* the described expression holds also for 72 hpf. On the other hand, *Pax3/7* was not detected in the episphere, whereas *Pax2/5/8* is expressed dorso-laterally.

Activation of Wnt/β-catenin by CHIR99021 from 24 to 48 hpf led almost complete loss of *Pdu-Pax6* from the hyposphere except from the most terminal cells, whereas it stays untouched in the episphere. Also the expression of *Pdu-Pax3/7* is much weaker, especially in the first segment. *Pdu-Pax2/5/8* diminishes from the episphere and its ventral border is shifted more laterally.

Inhibition of the pathway by OD270 in the same time window causes narrowing of the two longitudinal stripes of *Pdu-Pax6* expression in the ventral nerve cords and concomitantly the widening of the midline gap between them. The episphere expression in the larval eye region becomes restricted to narrow stripes along the prototroch. But this can be only the result of individual variability in the samples, since it is not supported by the other inhibitor IWR-1-endo where the episphere expression appears to be normal. On the other hand, the hyposphere expression domains of *Pdu-Pax6* are reduced and the midline gap even more widened. No major differences from the control can be observed after the treatment from 48 to 72 hpf. *Pdu-Pax3/7* is expressed even stronger at 48 hpf after 24 to 48 treatment with OD270, reaching slightly more medially and with less prominent segmental pattern. Inhibition of *Pdu-Pax2/5/8* is not included in the analysis.

The images are 3D projections of confocal *z*-stacks of fluorescent whole mount *in situ* hybridization. Approximate diameter of a 48 hpf larva after the hybridization procedure is around 130 µm, 72 hpf stage is to scale. Ventral views are oriented anterior up, lateral views with their ventral facing to the right. Representative individuals are shown.

10.2.3. Antero-posterior patterning

The neuroectoderm is patterned in the anterior to posterior direction by a sequential expression of the several homeobox transcription factors (section 7.2.3.3). The expression of these TFs has been described in *Platynereis* (Steinmetz et al., 2011; Steinmetz et al., 2010) but their potential regulation by Wnt/β-catenin signalling was not studied and remained unknown.

10.2.3.1. Anterior patterning genes of the episphere

The anterior-most tip of the developing neural tissue with the exception of apical organ (Marlow et al., 2014) is marked in most bilaterians by the expression of *Six3* (Steinmetz et al., 2010). It is followed (with some overlap) by still anterior expression of *Otx* which makes a sharp boundary with the posterior *Gbx* (see the section 7.2.3.3). This boundary is in *Platynereis* located behind the prostomium, i. e. approximately on the border between the episphere and hyposphere.

By the end of the trochophore/beginning of the metatrochophore stage at 48 hpf, *Pdu-Six3* is expressed in the almost entire episphere, with the exception of the vicinity of the prototroch, and in the part of the stomodaeum that is located anterior to the prototroch. Counterintuitively, the anterior marker *Pdu-Six3* expands from the episphere and is weakly expressed in the hyposphere upon the activation of Wnt/β-catenin by CHIR99021. However, it slightly retreats from the prototroch in the episphere. This is especially evident in the stomodaeum in which only the expression in the centre is maintained and ceases to be continuous with the *Pdu-Six3* expression in the rest of the episphere. After the inhibition of canonical Wnt signalling by IWR-1-endo*, Pdu-Six3* expression in the episphere similarly slightly retreats but is also reduced in its intensity – an unexpected situation in an anterior marker that was originally presumed to be repressed by a posterior Wnt signalling under normal circumstances.

The expression of *Pdu-Otx* in the neuroectoderm is broad but patchier than that of *Pdu-Six3*. It is present in most of the episphere, where these two genes overlap, in the stomodaeum and the peristomium as was described before (Arendt et al., 2001). In the ventral neuroectoderm of the hyposphere, *Pdu-Otx* copies the trajectory of VNCs, their commissures and displays a segmental pattern as well. Patches of *Pdu-Otx* expression are found also in the putative posterior dorsal chaetal sacs where *Pdu-WntA* and *Pdu-Wnt5* are also present (cf. the chapter 10.1.1) and in paired dorsal regions posterior to the prototroch.

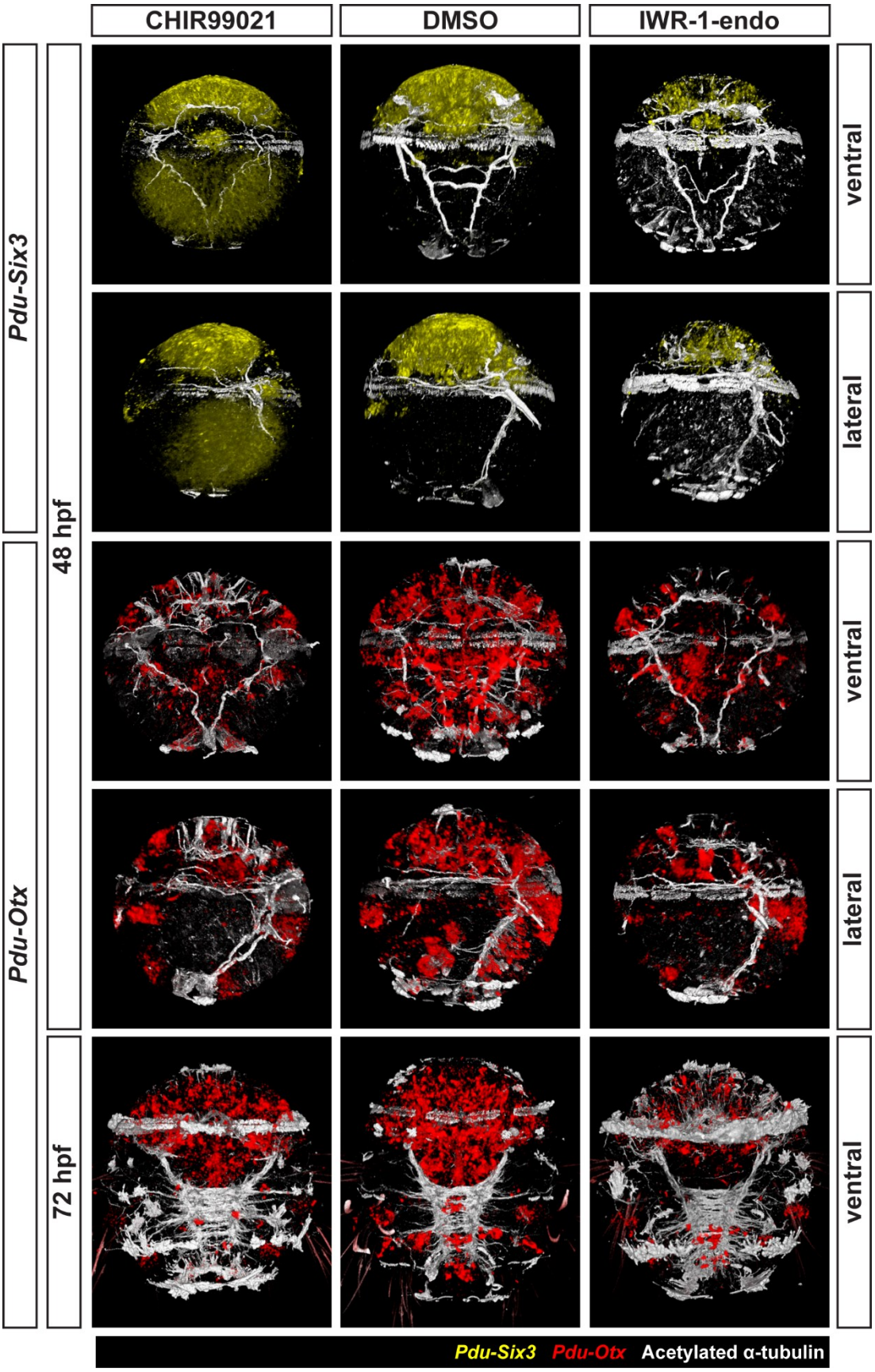


Figure 21 – The expression of anterior A-P patterning genes

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Figure 21 – The expression of anterior A-P patterning genes

The expression of *Pdu-Six3* and *Pdu-Otx*, the first two gene of the neuroectodermal A-P patterning system (Steinmetz et al., 2011; Steinmetz et al., 2010) in 48 hpf stage after pharmacological treatment from 24 to 48 hpf and in 72 hpf stage after the 48 to 72 hpf treatment.
Pdu-Six3 is expressed in most of the episphere including the anterior portion of the stomodeal rosette, except the ciliary belt in controls (DMSO). In CHIR99021 treated (with activated Wnt/β-catenin signalling) 48 hpf larvae, the posterior border of *Six3* expression in the episphere slightly shifts to the anterior, as expected if *Six3* were negatively regulated by posteriorly active Wnts. However, a novel weak expression appears ectopically in the hyposphere, contradicting this hypothesis. After inhibition of the canonical Wnt pathway, *Pdu-Six3* is also downregulated in the episphere.
At 48 hpf, *Pdu-Otx* is present in a band of ectoderm below the prototroch, in stomodaeum, as published (Arendt et al., 2001) and in many other cells in the episphere, including larval and future dorsal eye regions (which was not reported previously for this gene), and in the ventral nerve cord and developing chaetal sacs (also not shown before). In 48 hpf larvae with Wnt/β-catenin pathway activated by CHIR99021, *Pdu-Otx* showed overall lower level of expression and its domain around the prototroch almost disappeared. Inhibition by IWR-1-endo led to a similar phenotype with only the expression in the larval eyes and the stomodaeum left.
The images are 3D reconstructions of confocal *z*-stacks of fluorescent whole mount *in situ* hybridization; anterior is up, in lateral views ventral to the right. Approximate size of a 48 hpf larva is 130 μm, 72 hpf stage is to scale. Representative individuals are shown.

At 72 hpf, the expression of *Pdu-Otx* is widespread in the episphere and stomodaeum and confined to the VNCs commissures, the second pair of segmental nerves and a pair of unknown domains in the second segment that might be connected to nephridia.

The expression of *Pdu-Otx* is highly reduced after either the activation or inhibition of the Wnt/β-catenin signalling pathway. The expression in chaetal sacs is lost upon either activation or inhibition in agreement with the observation that chaetal sacs do not develop after Wnt/β-catenin overactivation (cf. with the chapter 10.3, Figure 24). On the other hand, the dorsal expression domains seem to be affected only by Wnt inhibition.

Unfortunately, I was not successful in the generation of a functional *in situ* hybridization probe for the other TF *Pdu-Gbx*, that could help to more precisely identify the exact position of the *Otx-Gbx* boundary that could be potentially homologous to the midbrain-hindbrain boundary of vertebrates.

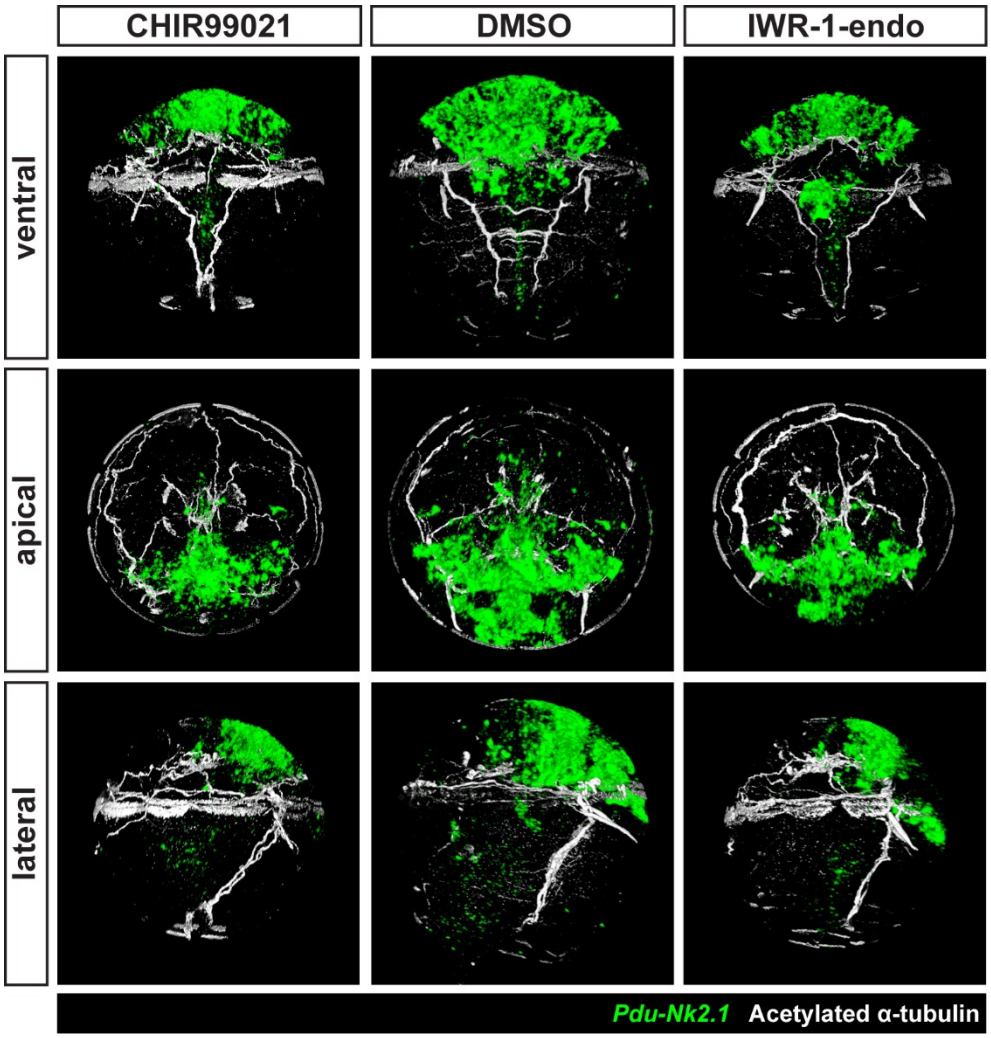

Figure 22 – Expression of *Pdu-Nk2.1*

Changes in the expression pattern of the *Pdu-Nk2.1* in 48 hpf larvae after pharmacological treatment of Wnt/β-catenin pathway from 24 hpf. Under normal conditions (control, DMSO), *Pdu-Nk2.1* marks ventromedial neuroectoderm in the episphere. It extends a medial domain apically where it abuts apical organ. It is also strongly expressed in the stomodaeum and continues as a narrow strip of weak expression in the ventral midline.

Activation of Wnt/ β-catenin pathway by CHIR99021 leads to a loss of stomodeal expression and the reduction of medial apical domain around the apical organ. Interestingly, only the two apical cells expressing *Pdu-Nk2.1* close to crescent cells mentioned by Marlow et al. (2014) are left.

Inhibition by IWR-1-endo leaves the *Nk2.1* pattern slightly reduced, especially evident in the smaller stomodeal expression domain.

The images are 3D reconstructions of confocal *z*-stacks of *Pdu-Nk2.1* fluorescent whole mount *in situ* hybridization. Approximate size of a 48 hpf larva after *in situ* hybridization procedure is around 130 µm. Larvae from ventral views are positioned with their anterior to the top, apical views are oriented ventral down. Representative individuals are shown.

Pdu-Nk2.1 belongs to the medio-lateral patterning system as the vertebrate *Nkx2.1* is via the negative regulation by dorsal Wnt/β-catenin signalling restricted to the ventral telencephalon (Backman et al., 2005). Nevertheless, I decided to include it here because of its quite similar and overlapping expression with the preceding two genes in *Platynereis*. I analysed the *Pdu-Nk2.1* expression in the 48 hpf stage (Figure 22) and found out that it is expressed in a cross-like domain in the ventral episphere, i. e. the stomodaeum, the neuro-ectoderm of the ventral cerebral ganglia and the surroundings of the apical organ. Upon Wnt/β-catenin activation, the stomodaeal domain is lost and the expression in the other arms of the "cross" is reduced. Likewise, the *Pdu-Nk2.1* expression domain is smaller also upon the inhibition of Wnt/β-catenin signalling by IWR-1-endo with the difference that the stomodaeal *Pdu-Nk2.1* domain persists but becomes separated from the rest of the expression. There is also another site of *Pdu-Nk2.1* expression which is, however, very faint. It reminds a plane located in the midline between the left and right halves of the hyposphere, probably on the contact between the macromeres. It is present, although reduced in size, in either Wnt activation or inhibition and could be explained by unspecific trapping of the probe unless I did not observe it with any other *in situ* hybridization RNA probe.

10.2.3.2. A-P patterning of the ventral neuroectoderm by Hox genes

The differentiation along the A-P axis is posterior to the MHB (in vertebrates) or in the hyposphere (in *Platynereis*) achieved by a A-P nested or overlapping pattern of *Hox* genes (cf. the sections 7.2.2.4, 7.2.3.3 and 7.3.7.1). In Platynereis, *Pdu-Hox1*, *Pdu-Hox4* and *Pdu-Lox5* have their anterior boundaries in the segments I, II and III, respectively.

Either *Pdu-Hox1* and *Pdu-Hox4* are expressed as a broad transversal stripe in ventral and reaching to lateral ventral (neuro)ectoderm in the early metatrochophore at 48 hpf (Figure 23). The expression of *Pdu-Hox1* is severely reduced upon either activation (CHIR99021) or inhibition (IWR-1-endo) of Wnt/β-catenin pathway but the traces of *Pdu-Hox1* transcripts can be found in the correct location. Similarly, *Pdu-Hox4* is also reduced upon both types of pharmacological modulation of Wnt/β-catenin pathways activity, although it is expressed quite well even in the presence of the Wnt activator but not in its medial-most domain. It is again almost completely absent after the treatment with Wnt inhibitor, but in both cases the remaining *Pdu-Hox4* expression can be found in the correct location. This observation is confirmed for both genes and also for *Pdu-Lox5* by prolonged

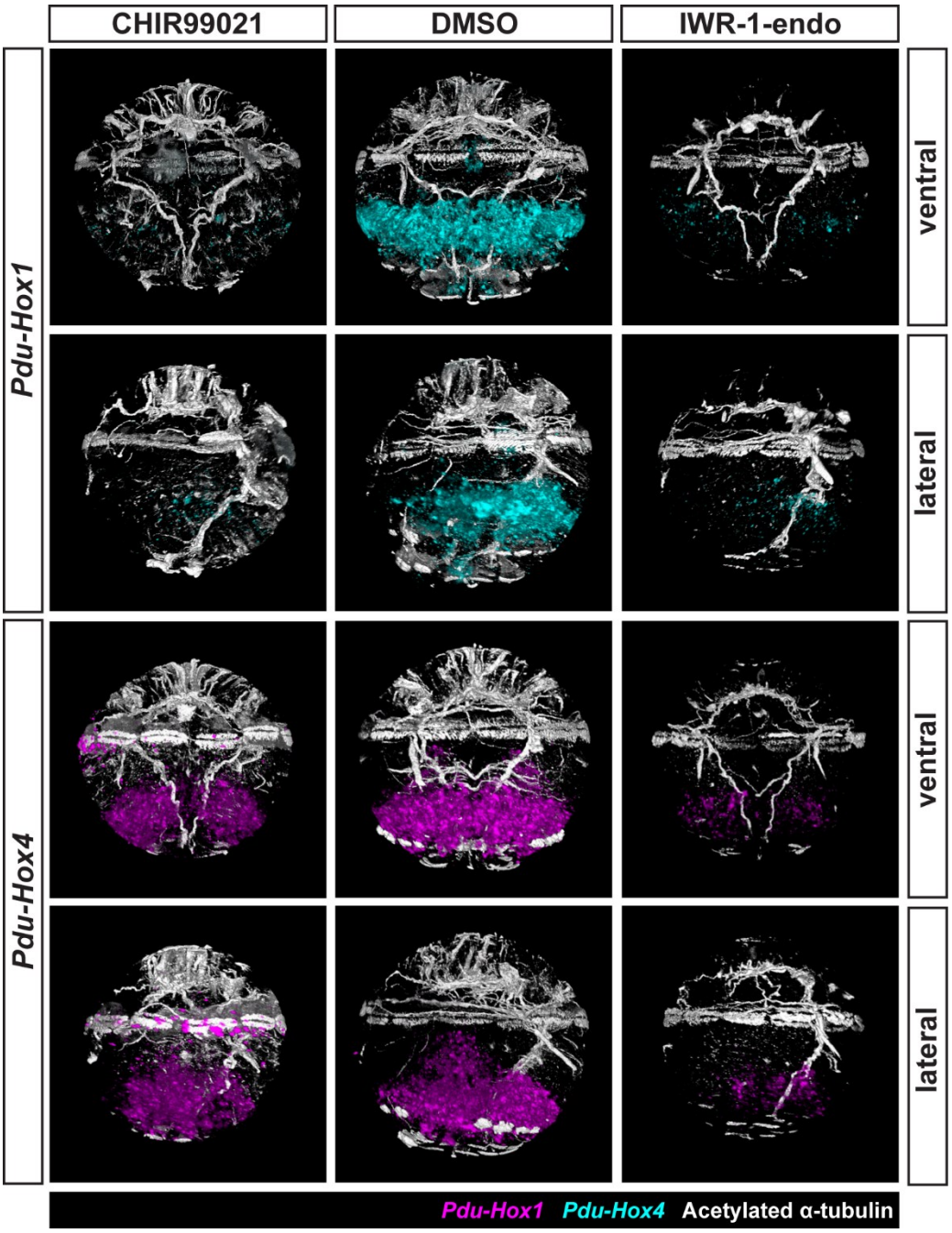

Figure 23 – Expression of *Hox* genes at 48 hpf

Under normal circumstances (DMSO control), *Hox* genes *Pdu-Hox1* and *Pdu-Hox4* are expressed in broad transversal bands of neuroectoderm in a staggered anterior-posterior fashion in the hyposphere of 48 hpf larva. *Pdu-Hox1* is expressed more anteriorly, with the anterior boundary of expression reaching almost the first axonal commissure between the two ventral nerve cords, while *Pdu-Hox4* is located more posteriorly reaching only the second commissure.
Both activation (by CHIR99021) or inhibition (by IWR-1-endo) from 24 to 48 hpf caused downregulation of *Hox* levels but did not alter their spatial pattern.

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(Figure 23 – continuation from the previous page)

The images are 3D reconstructions of confocal fluorescent whole mount *in situ* hybridization *z*-stacks. Approximate size of a 48 hpf larva after hybridization procedure is around 130 μm. Ventral views are oriented with anterior to the top, lateral views with ventral to the right. Representative individuals are shown

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treatments from 24 to 72 hpf and analysis at 72 hpf (Figure 25) done primarily for the study of segmentation, when all these genes are expressed although in lower levels in a correct location along the A-P axis of the ventral neuroectoderm. In conclusion, it seems that the *Platynereis Hox* genes are not directly regulated by Wnt signalling.

10.3. Segmentation

Based on the expression of *Pdu-Wnt* genes, *Pdu-Engrailed* and the study of Hedgehog pathway, it was proposed that the Wnt/β-catenin signalling could be engaged in the establishment of segment boundaries in *Platynereis* in a mechanism similar to the one found in insects (section 7.3.7.2). In insects, Wnt proteins produced on the posterior edge of one body segment activate Wnt/β-catenin signalling in the anterior row of cells of the following parasegment which activates the expression of *Engrailed* and *Hedgehog*, that in turn upregulate *Wnt* in the anterior parasegment (see the section 7.2.2.2 for more detailed description with references). However, the role of Wnt/β –catenin signalling in the segmentation process and the regulation of segmentation genes was not demonstrated by any functional study.

In order to verify whether the Wnt/β-catenin signalling controls also the expression of *Engrailed* on the intersegmental boundary in *Platynereis,* I detected the expression of *Pdu-En* by whole mount *in situ* hybridization on larvae of *Platynereis* that were the prior to fixation treated with the Wnt activator CHIR99021 or inhibitor IWR-1-endo (Figure 24). *Pdu-En* was detected in a segmental pattern in the ventro-lateral and lateral ectoderm of the control 48 hpf larvae treated with DMSO from 24 hpf. The expression in most segmental stripes does not reach medially to the VNCs. Upon the ubiquitous activation of Wnt/β-catenin pathway, the segmental pattern of *Pdu-En* becomes even more pronounced. The segmental stripes now reach medially up to the VNCs, are much stronger and extend from the ectoderm deeper into the tissue so that thy create whole parallel sheets of *Pdu-En*

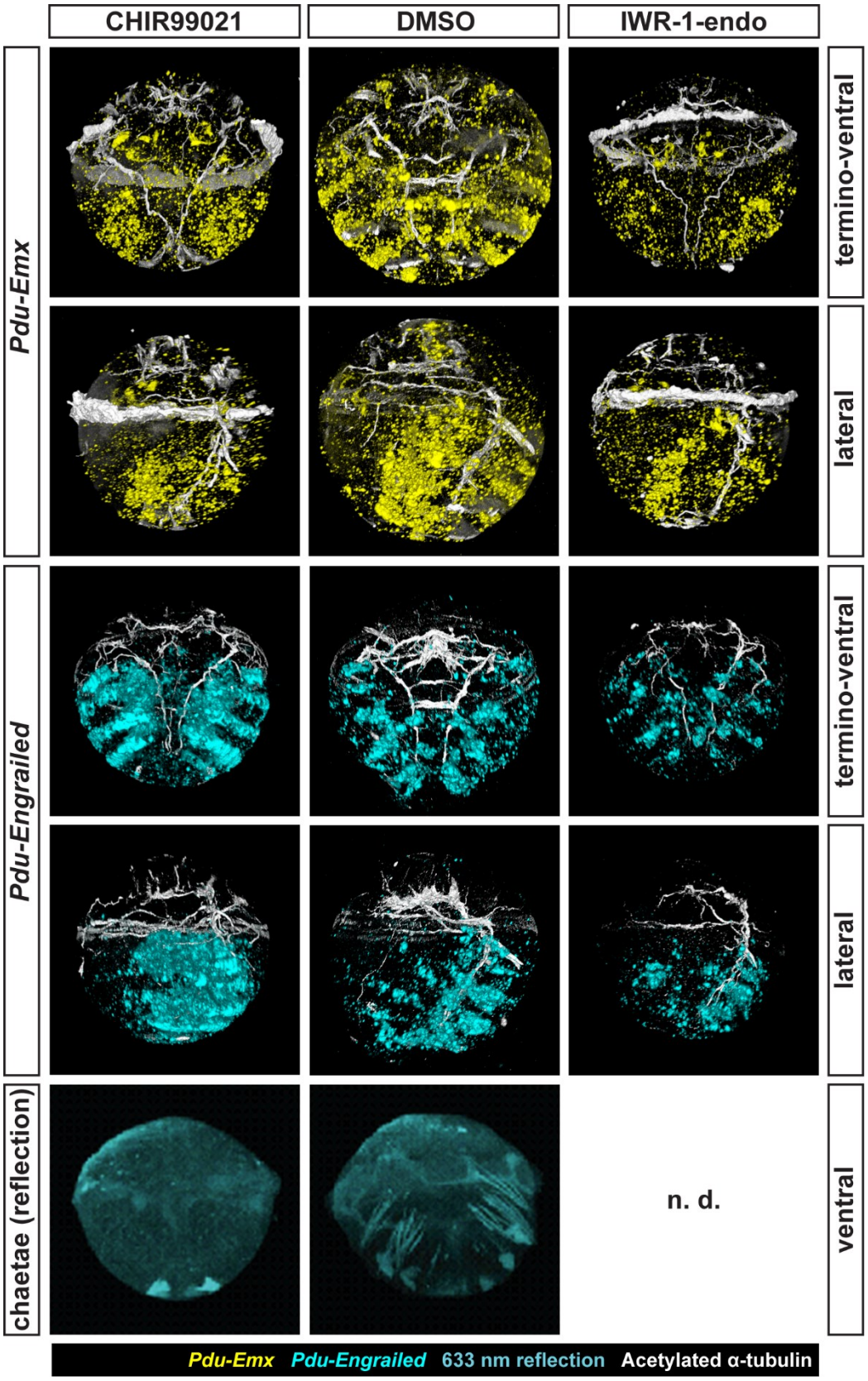

Figure 24 – Segmental expression of *Pdu-Emx* and *Pdu-Engrailed* at 48 hpf

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Figure 24 – Segmental expression of *Pdu-Emx* and *Pdu-Engrailed* at 48 hpf

Pdu-Emx is expressed in two small bilaterally symmetrical regions in the episphere as reported before (Tomer et al., 2010) but much broader expression is found in the hyposphere where *Pdu-Emx* is present in the striking segmental pattern and in lateral domains, probably the developing chaetal sacs.
The segmental distribution of *Pdu-Emx* disappears when the Wnt/β-catenin signalling is activated by CHIR99021 or inhibited by IWR-1-endo, suggesting that in either situation the segments are not properly formed. The brain-specific expression in the episphere is lost in both conditions (nearby signal is an unspecific staining on the surface of lipid droplets in macromeres). Neither of the effects could be seen with OD270, probably because it is too weak or it did not work well on this batch (due to the precipitation from water etc.).
Pdu-Engrailed is normally (DMSO control) expressed in segments. Interestingly, unlike *Pdu-Emx*, the segmental pattern of *Pdu-Engrailed* is retained after activation of canonical Wnt signalling by CHIR99021 and the expression is even broader and stronger. This implies a positive regulation of *Pdu-Engrailed* by Wnt signalling alternating with a strong inhibition of Wnt/β-catenin signalling or its activation of *Pdu-Engrailed* [probably by Hedgehog (Dray et al., 2010)].
Chaetae visualized by reflection of 633 nm laser as described by Zheng et al. (2011). Despite preserved striped pattern of *Pdu-Engrailed*, chaetae (mesodermal structures) do not develop after activation of Wnt/β-catenin pathway.
The images are 3D projections of confocal *z*-stacks of fluorescent whole mount *in situ* hybridization or 633 nm laser reflection. Approximate size of a 48 hpf larva is around 130 μm. *Pdu-Emx* and *Pdu-Engrailed*: Termino-ventral views oriented with anterior to the top, lateral views with ventral to the right; *reflection*: ventral views. Representative individuals are shown.

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Figure 25 – Expression of *Hox* genes and *Pdu-Engrailed* are not changed after prolonged treatment of Wnt/β-catenin pathway

Larvae treated from 24 to 72 hpf, when the morphological segments develop, with the activator (CHIR99021) or inhibitor (IWR-1-endo) of Wnt/β-catenin signalling cascade and fixed at 72 hpf. Although morphological segments fail to form, *Hox* genes are still expressed in correct domains and sequence. Also *Pdu-Engrailed* is still expressed segmentally upon Wnt/β-catenin activation whereas with IWR-1-endo inhibitor, it shows only traces of segmental pattern.
The images are 3D projections of confocal *z*-stacks of whole mount *in situ* hybridization staining. The larvae are oriented with their anterior facing to the top. Representative individuals are shown.

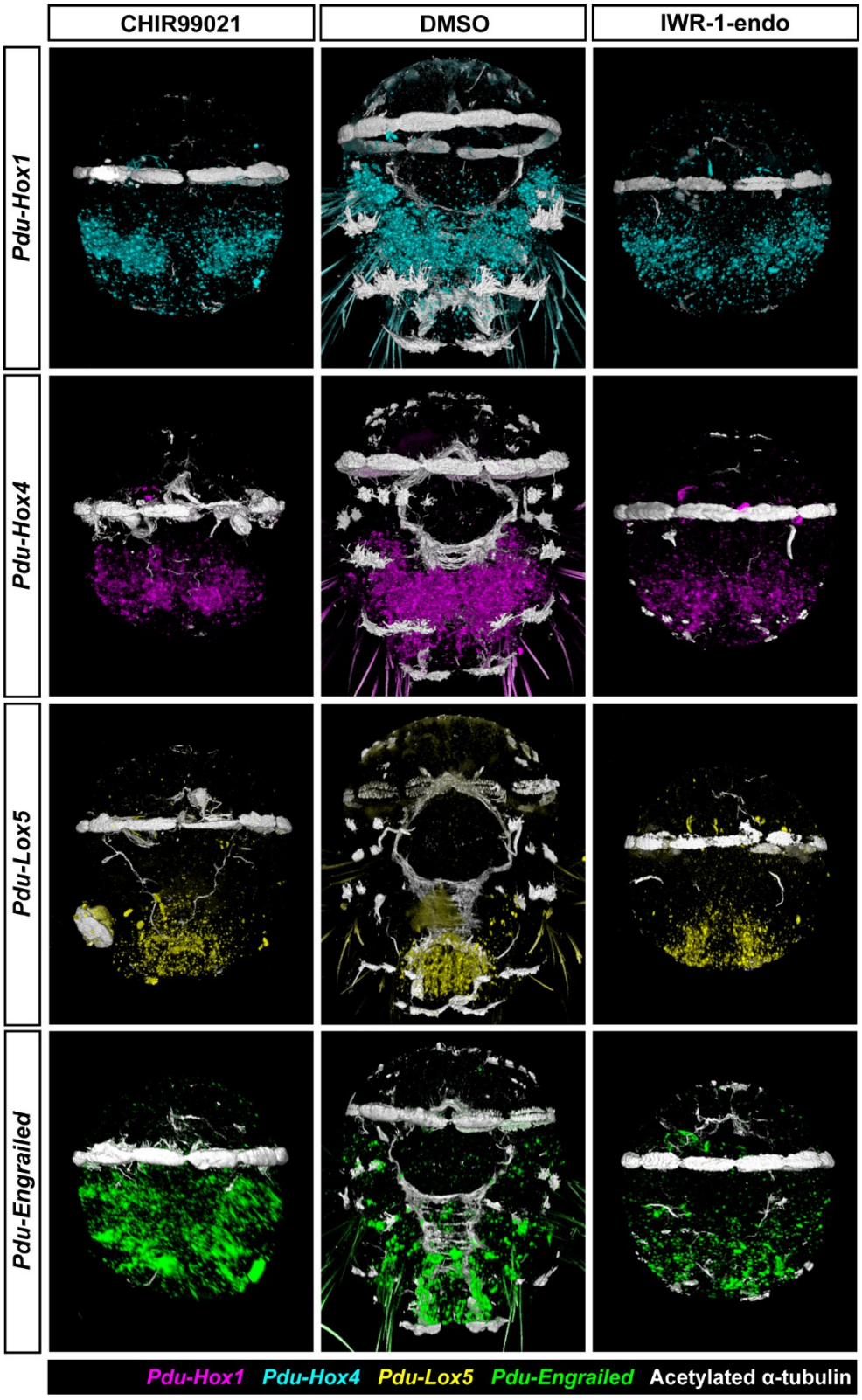

Figure 25 – Expression of *Hox* genes and *Pdu-Engrailed* are not changed after prolonged treatment of Wnt/β-catenin pathway

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positive cells. On the other hand, the inhibition of canonical Wnt signalling by IWR-1-endo results in weaker *Pdu-En* expression; nevertheless, in still recognizable segmental stripes. After a prolonged treatment from 24 to 72 hpf, *Pdu-En* is expressed ectopically with only hints of segmental stripes following the activation of Wnt/β-catenin signalling, whereas its expression is scarce and disorganized after the inhibition. Taken together, Wnt/β-catenin signalling positively regulates *Pdu-En* on the intersegmental boundary but it can do so only in the permissive region in the previously established segmental stripes of *Pdu-En.*

Other TFs that participate on the patterning of neuroectoderm also display segmental expression. It is the case of *Pdu-Otx*, which was discussed in the previous chapter (section 10.2.3.1), and of another neurospecific TF *Pdu-Emx* (Figure 24). The latter is expressed in bilateral domain on both sides of the apical organ, in segmental stripes of the ventral neuroectoderm and in the developing chaetal sacs. The episphere expression is lost upon either activation or inhibition of Wnt/β-catenin signalling. The expression in the hyposphere is highly reduced in both conditions, similar to *Pdu-Otx* (Figure 21). Remaining *Pdu-Emx* transcripts are disordered and partly lose their segmental organization.

In addition to changes in gene expression, I also observed that the ectopic activation of Wnt/β-catenin pathway also blocks the development of first visible morphological structures – the metatrochs and the chaetal sacs (Figure 24). Given the overall similarity of the effect of activation and inhibition of canonical Wnt signalling on phenotype and gene expression of the treated larvae, a similar effect could be probably found also after a treatment with inhibitor, but this was not investigated. I was curious whether the excessive and/or ectopic Wnt/β-catenin signalling disrupts the morphogenesis of segments or the lack of segmental structures might be caused by a loss of identity of segments, probably via the deregulation of *Pdu-Hox* gene expression.

As mentioned in the previous chapter, section 10.2.3.2, the treatment with either activator or inhibitor of Wnt/β-catenin signalling from the hour 24 to 48 of development severely reduces the intensity of expression of *Pdu-Hox* genes at 48 hpf but does not shift the boundaries of the remaining expression domains. The development of first segmental structures, the metatrochs and chaetal sacs, takes place in this time window but morphologically distinct segments are formed later. I thus performed a prolonged pharmacological treatment with the activator CHIR99021 and inhibitor IWR-1-endo from the onset of the trochophore stage at 24 hpf to the late metatrochophore stage (72 hpf) to cover the entire period of morphological formation of segments. All three investigated *Pdu-Hox* genes (*Pdu--Hox1*, *Pdu-Hox4* and *Pdu-Lox5*) apparently retain their expression in the

area that corresponds to the proper segment and therefore, there was no homeotic shift. However, the morphological formation of segments and segmental structures is obviously abrogated which results in the overall developmental arrest. This is evidenced by a lack of intersegmental grooves or septa, chaetal sacs, segmental nerves and transversal commissures between the VNCs and reduced development of cerebral ganglia, ciliary belts (metatrochs and akrotrochs), i. e. basically all observable structures that develop since 24 hpf, including non-segmented ones.

These results demonstrate that at least in this time period, Wnt/β-catenin signalling does not regulate the expression of *Hox* genes and hence the segment identity. However, this does not rule out that it is required for the onset of *Pdu-Hox* expression earlier in development. During the trochophore and metatrochophore stages (24 to 72 hpf), Wnt/β-catenin signalling is involved in the establishment of segment boundaries by a positive regulation of *Pdu-Engrailed* and tightly regulated signalling is necessary for proper morphogenesis of segmental structures.

10.4. Gut development

10.4.1. Digestive enzymes and compartmentalization of the gut

Out of the all domains of *Pdu-Tcf* and *Pdu-Axin* expression and β-catenin protein localization during development, the most attention drew the striking simultaneous appearance of all these three key components of the Wnt/β-catenin pathway in the developing midgut of the nectochaete larvae between the days 5 and 7 of development.

In order to gain insight into the function of Wnt/β-catenin signalling in the developing gut of 7 days old larvae, I first asked myself following questions:

1. Do the gut compartments differentiate properly?
2. What tissue of the gut requires the activity of Wnt/β-catenin signalling – the gut endoderm or the surrounding mesoderm?

To answer both these questions at once, I chose to examine several digestive enzymes which function in the endodermal digestive epithelium and were previously reported to be specific for certain gut compartments (Williams et al., 2015). They were the protease *Pdu-Enteropeptidase*, the peptidases *Pdu-Subtilisin-1* and *Pdu-Subtilisin-2*, the polysaccharide-digesting enzyme *Pdu-α-Amylase* and the intracellular digestive enzyme *Pdu-Legumain-protease-precursor* (hereafter as *Pdu-Legumain*). I performed pharmacological treatments of Wnt/β-catenin pathway with the activator CHIR99021 or inhibitors IWR-1-endo and JW55 in the nectochaete stage between the days 5 and 7 of development. In this time period, the midgut cellularizes and establishes the expression of *Pdu-Tcf* and *Pdu-Axin* and high levels of β-catenin. I then performed the fluorescent whole mount *in situ* hybridization on these larvae with digoxigenin-labelled antisense RNA probes which were specific to mRNAs of the aforementioned digestive enzymes to reveal possible changes in their expression upon the modulations of Wnt/β-catenin pathway (Figure 26A).

The development already starts to be asynchronous by the end of the nectochaete stage and each treatment thus produced a range of phenotypes. To accurately assess whether the compound and the consequent modulation of the activity of Wnt/β-catenin pathway has an effect on the expression of genes at this stage, I scored the intensity and/or localization of expression in all larvae after the end of *in situ* hybridization procedure by bright field microscopy (Figure 26B).

Pdu-Enteropeptidase is expressed in small bilateral patches (possibly glands) in the pharynx and its expression was not changed upon any of the Wnt treatments (not shown).

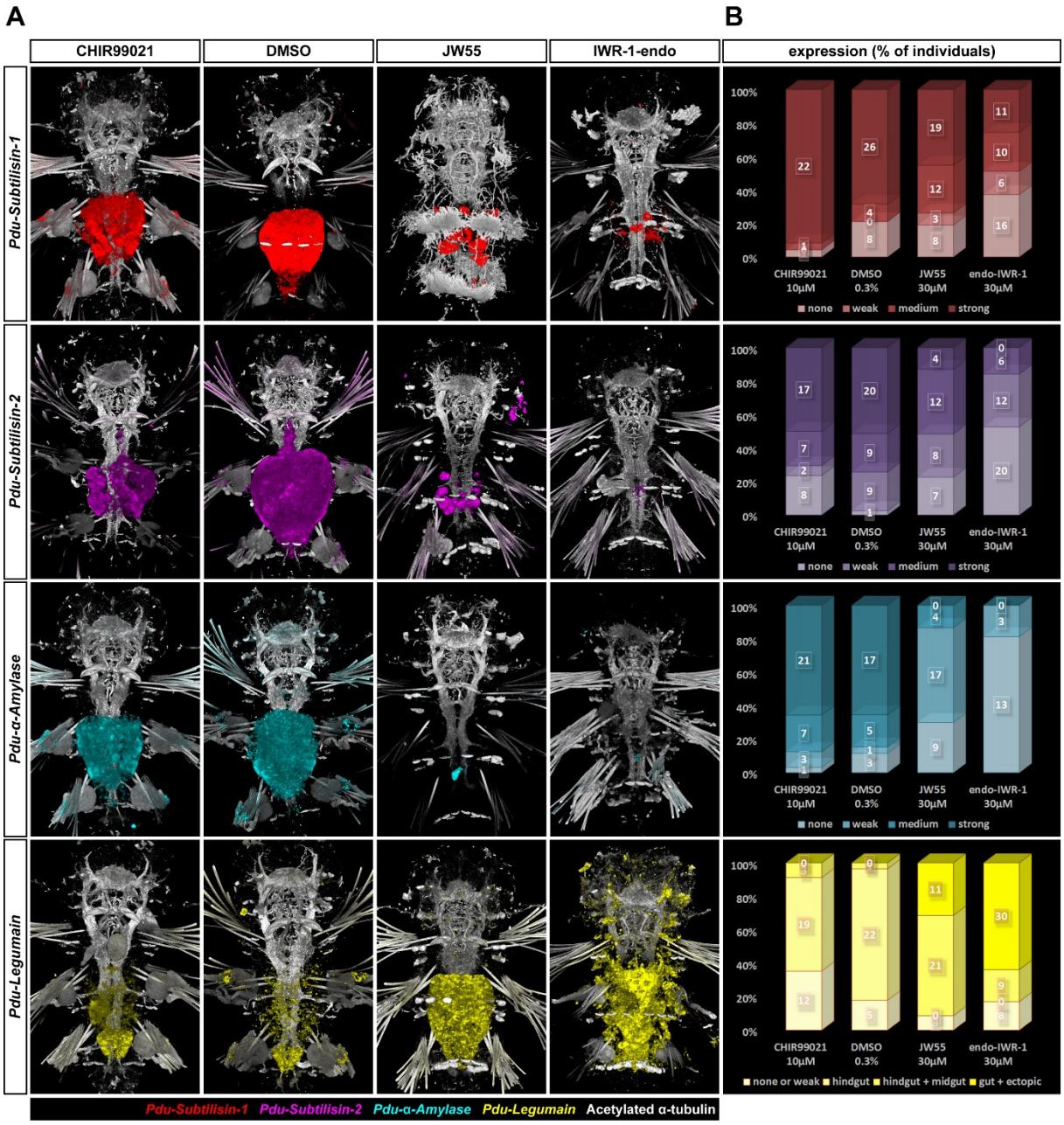

Figure 26 – Inhibition of Wnt/β-catenin pathway converts the midgut to the hindgut

(A) Fluorescent *in situ* hybridization of digestive enzymes which are markers of midgut (*Pdu-Subtilisin-1*, *Pdu-Subtilisin-2, Pdu-α-Amylase*) or hindgut (*Pdu-Legumain*).
Pdu-Subtilisin-1 – first row, *red*; *Pdu-Subtilisin-2* – second row, *magenta*; *Pdu-α -Amylase* – third row, *cyan*; *Pdu-Legumain* – fourth row, *yellow*. Representative individuals are shown.
(B) Quantification of Wnt/β-catenin pathway activation/inhibition on the expression of these genes by assignment of all individuals to phenotypic classes. The darker the colour in the graph, the higher the expression. All three midgut marker genes show significantly lower or no expression upon inhibition Wnt/β-catenin signalling pathway by JW55 or IWR-1-endo. Activation of the pathway

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(Figure 26 – continuation from the previous page)

(CHIR99021) produced no significant changes in gut expression of these genes. *Pdu-Legumain* is highly expressed in the hindgut but only in very low levels in the midgut under normal circumstances (DMSO). This is also true for the activating condition. However, it expands from the hindgut to midgut upon Wnt/β-catenin inhibition by either IWR-1-endo and JW55. Moreover, stronger inhibitor (IWR-1-endo) causes *Pdu-Legumain* to be expressed ectopically outside the digestive tract in nephridia and other cells.
The images are 3D projections of confocal *z*-stacks of whole mount *in situ* hybridization staining on 7 dpf *Platynereis* larvae treated with chemical activator or inhibitors of Wnt/β-catenin signalling from 5 dpf. All images are dorsal views with their anterior oriented to the top.

Under normal conditions with unaffected Wnt signalling (DMSO control; Figure 26A – second column from the left), the 7 days old *Platynereis* larvae strongly express *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2* and *Pdu-α-Amylase* in their midguts. Only more or less weak expression of these genes could be found in the hindguts of some, but not all specimens. On the other hand, a strong expression of *Pdu-Legumain* is restricted to the hindgut and the expression in the midgut is almost undetectable. I thus considered *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2* and *Pdu-α-Amylase* as the midgut marker genes, whereas *Pdu-Legumain* to be the marker of hindgut.

The inhibition of Wnt/β-catenin signalling by CHIR99021 quite surprisingly had no major effect on nor the expression nor localization of any of these four genes (Figure 26A – the first column from the left, quantification on the Figure 26B). Conversely, the inhibition of Wnt/β-catenin signalling by either JW55 or IWR-1-endo caused the mRNA of the midgut-specific digestive enzymes *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2* and *Pdu-α-Amylase* to be reduced or even completely diminish from the midgut. On the other hand, the strong expression of hindgut marker gene *Pdu-Legumain* expanded from the hindgut to the midgut upon milder inhibition of Wnt/β-catenin pathway by JW55 or even beyond the gut to the larval nephridia and ectoderm with the stronger Wnt inhibitor IWR-1 endo (Figure 26A– the third and fourth columns, quantification on the Figure 26B). It also proves that the gut is present after the inhibition of Wnt/β-catenin signalling and the loss of expression of other enzymes upon Wnt inhibition is not due to the absence of the gut.

Wnt/β-catenin signalling in the gut thus seems to have a necessary permissive function for the development of the digestive epithelium of the midgut and its inhibition causes the midgut epithelium to assume hindgut-like characteristics.

10.4.2. Neurospecific genes in gut development

Some transcription factors that participate on the regionalization of the developing nervous system are also active during the specification of endoderm. This is the case of *Nk2.1* and *Otx* (cf. the sections 7.2.3.2 and 7.2.3.3 with the section 7.2.4.1). Therefore, although I already described their expression and regulation by Wnt/β-catenin signalling during the patterning of neuroectoderm in *Platynereis* (section 10.2.3.1), I investigated them again in the period of midgut development.

10.4.2.1. Pdu-Nk2.1

The transcription factor Nk2.1 is specifies neural fates in the medial/ventral nervous system (section 7.2.3.2), but it is also involved in the specification of the endoderm (section 7.2.4.1) and its orthologue is expressed in both midgut and hindgut of another annelid *Capitella teleta* (Boyle et al., 2014). Therefore, I was curious whether it is present also in the developing gut of *Platynereis* and if will also respond to the manipulations of canonical Wnt signalling.

I found out that *Pdu-Nk2.1* is expressed in the middle of the head of the 7 days old nectochaete stage and in two smaller regions on the sides of the head. This expression, although interesting, was not of my interest now. More importantly, *Pdu-Nk2.1* is also present in both the developing midgut and hindgut (Figure 27A – second image from the left).

Neither the activation of Wnt/β-catenin pathway by CHIR99021 or its inhibition by JW55 or IWR-1-endo caused any change in the *Pdu-Nk2.1* expression, which continues to be expressed in either the midgut and hindgut compartments (Figure 27A; quantification Figure 27B). This indicates that *Pdu-Nk2.1* is a more general endodermal marker which seems not to be regulated by Wnt/β-catenin signalling or does not require Wnt signalling for its expression at this stage anymore. As it is under normal circumstances expressed in either the midgut and the hindgut compartments, its expression pattern naturally would not be affected by a proposed midgut-to-hindgut conversion upon an inhibition of Wnt/β-catenin pathway. The observations of *Pdu-Nk2.1* are thus not in conflict with the role of Wnt/β-catenin in the discrimination of the midgut from the hindgut. *Pdu-Nk2.1* expression also further proves that an endodermal gut is present after Wnt/β-catenin pathway inhibition.

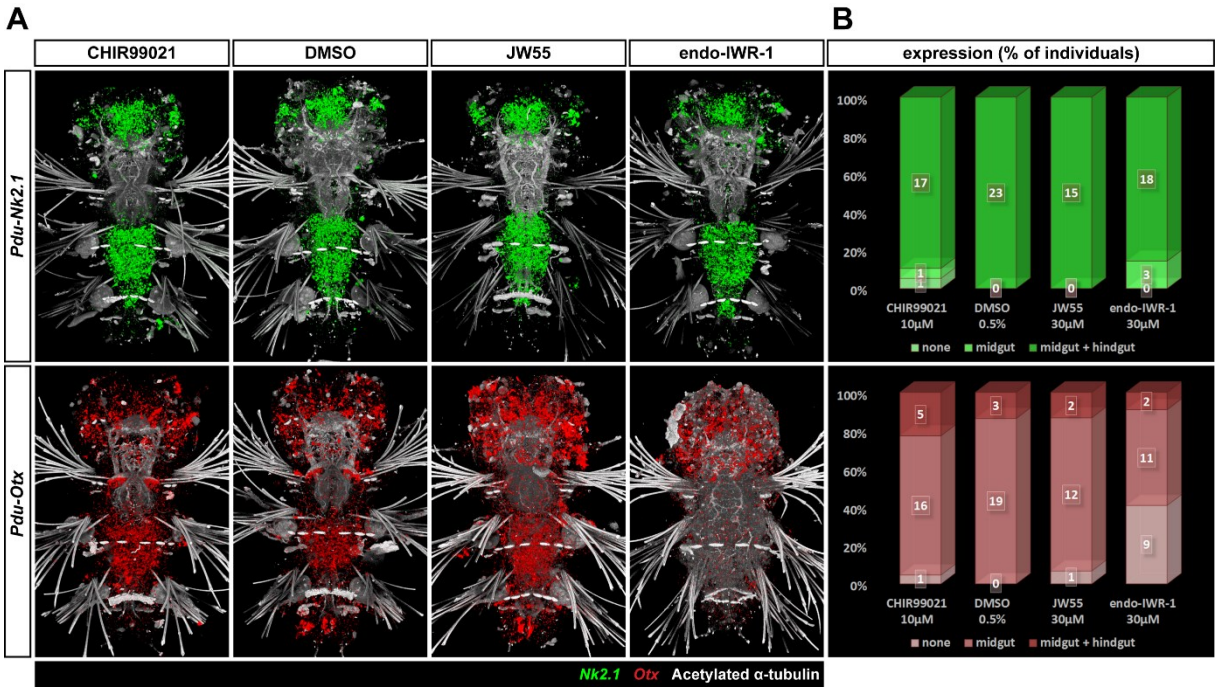

Figure 27 – The expression of neural-specific transcription factors in the endoderm

(A) 3D projections of confocal *z*-stacks of fluorescent *in situ* hybridization staining for two endodermal marker transcription factors, *Pdu-Nk2.1* (top row, *green*) and *Pdu-Otx* (bottom row, *red*) on 7 dpf *Platynereis* larvae treated from 5 to 7 dpf. These transcription factors are specific for neuroectoderm early in development but (similar to *Pdu-Tcf*) they appear in new expression domains in the gut endoderm later in development. At the same time, they continue to be expressed in the brain.

Pdu-Nk2.1 is present in both midgut and hindgut under normal circumstances and the same pattern is observed in the presence of any of the compounds (be it activator or inhibitor) tested. This is in line with the hypothesis of midgut to hindgut conversion upon Wnt/β-catenin signalling inhibition since the gene is normally expressed in both aforementioned gut compartments.

Pdu-Otx is present in mandibular cells and the midgut, whereas it is absent from most of the hindgut except for the very posterior cells that form the sides of the anus. The stronger inhibitor (IWR-1-endo) causes the midgut expression of *Pdu-Otx* to disappear, while the normal pattern is observed with the milder inhibitor JW55 (which just might suggest that this inhibitor did not work well on this batch of larvae). Neural expression is preserved under all conditions. The mandibular domains of *Pdu-Otx* are somewhat reduced after Wnt/β-catenin inhibition due to (or causing) the overall smaller size of the mandibles. Interestingly, the cells surrounding the anus lose *Pdu-Otx* expression in the presence of either activator or inhibitors of Wnt/β-catenin signalling.

All images are dorsal views oriented with anterior to the top.

(B) Quantification of the effect of Wnt/β-catenin signalling activation/inhibition on expression of these genes by assigning all individuals to phenotypic classes.

10.4.2.2. Pdu-Otx

The transcription factor Otx participates on the specification of the medial part of the brain and of the midbrain-hindbrain boundary in vertebrates (section 7.2.3.3) and is expressed in a complex pattern in the neuroectoderm of *Platynereis* metatrochophore (section 10.2.3.1). But it is also involved in the specification of endoderm and later marks the developing mouth (section 7.2.4.2). It is expressed in the stomodaeum and peristomium in *Platynereis* metatrochophore (Arendt et al., 2001) and in all parts of the developing gut in *Capitella* (Boyle et al., 2014).

In the 7 days old nectochaete stage of *Platynereis*, *Pdu-Otx* mRNA expression (Figure 27A) creates a complex pattern in the head, similar to what is observed in the epispheres of metatrochophore *Platynereis* larvae (section 10.2.3.1). Remarkable is the prominent expression in the jaws which represents the only remnant from the original stomodaeal domain in the metatrochophore stage. It is on the other side of the alimentary canal mirrored by a pair of cells with strong *Pdu-Otx in situ* hybridization signal on both sides of the anus. Most importantly, *Pdu-Otx* is also present in the midgut, but not hindgut.

The activation of Wnt/β-catenin signalling by CHIR99021 did not alter the expression patterns of *Pdu-Otx* in the head, jaws or the midgut, but interestingly interfered with its expression in the two cells on the sides of the anus.

The inhibition of Wnt/β-catenin pathway by JW55 did not cause any change and probably did not work on this particular batch of larvae. In contrast, the inhibition of canonical Wnt signalling by IWR-1-endo led to an absence of *Pdu-Otx* from the midgut, jaws and paired anal cells in a significant proportion of the larvae, while the head expression remained largely untouched. This situation suggests that the midgut loses its characteristic expression upon an inhibition of Wnt/β-catenin signalling pathway. Given that *Pdu-Otx* is in normal conditions expressed in the midgut, but not in the hindgut of *Platynereis* nectochaete larvae, it is also in agreement with the hypothesis that this leads to a conversion of the midgut digestive epithelium to a hindgut-like tissue.

10.4.3. ParaHox genes

ParaHox genes form a cluster with spatially collinear anterior-posterior expression similar to *Hox* genes. They play a crucial role in the antero-posterior patterning of the alimentary canal in vertebrates (section 7.2.4.2), especially *Cdx* genes that are direct targets of Wnt/β-catenin signalling (chapter 7.1.5). *ParaHox* genes are expressed in a collinear A-P pattern also in the developing gut of *Platynereis* (Hui et al., 2009). They thus represent good candidates for the regulators of the A-P identity of the gut compartments that could be regulated by Wnt/β-catenin signalling.

I prepared *in situ* hybridization probes for all three *Platynereis ParaHox* genes *Pdu-Gsx*, *Pdu-Xlox* and *Pdu-Cdx*. The former two were not able to detect the gene expression in fluorescent *in situ* hybridization. The *Pdu-Gsx* probe eventually did work in at least in standard non-fluorescent *in situ* hybridization but I did not investigate it after the manipulation of Wnt pathway and therefore, it is not presented here.

10.4.3.1. Pdu-Cdx/Caudal

The homeobox transcription factor Cdx/Caudal is necessary for the proper differentiation of the posterior part of the digestive tube (section 7.2.4.2) and of the body in general and the posterior growth in the specialized posterior growth zone (section 7.2.2.1). In *Platynereis*, it has been described previously to be expressed around the posterior and medial blastopore, in the pygidium, the segment addition zone (de Rosa et al., 2005) and the hindgut (Hui et al., 2009).

A high-resolution fluorescent *in situ* hybridization with the same antisense RNA probe previously used by Hui and colleagues revealed a broader expression pattern of *Pdu-Cdx* in the larvae of *Platynereis* at 7 dpf (Figure 28A, C – second panel of images from the left). Besides the midgut and pygidium, it is expressed also in the ventral midline of the gut that connects the hindgut domain with another on the midgut/foregut boundary (visible also on in the stainings of Hui et. al on the 6 dpf stage).

The anterior boundary of strong *Pdu-Cdx* mRNA expands anteriorly from the hindgut upon the activation of Wnt/β-catenin pathway by CHIR99021 (Figure 28A, C– first panel of images from the left). The domain on the midgut/foregut boundary is enlarged laterally and the stripe of *Pdu-Cdx* connecting it with the expression in the hindgut widens substantially. However, a somewhat weaker expression is present across the entire midgut.

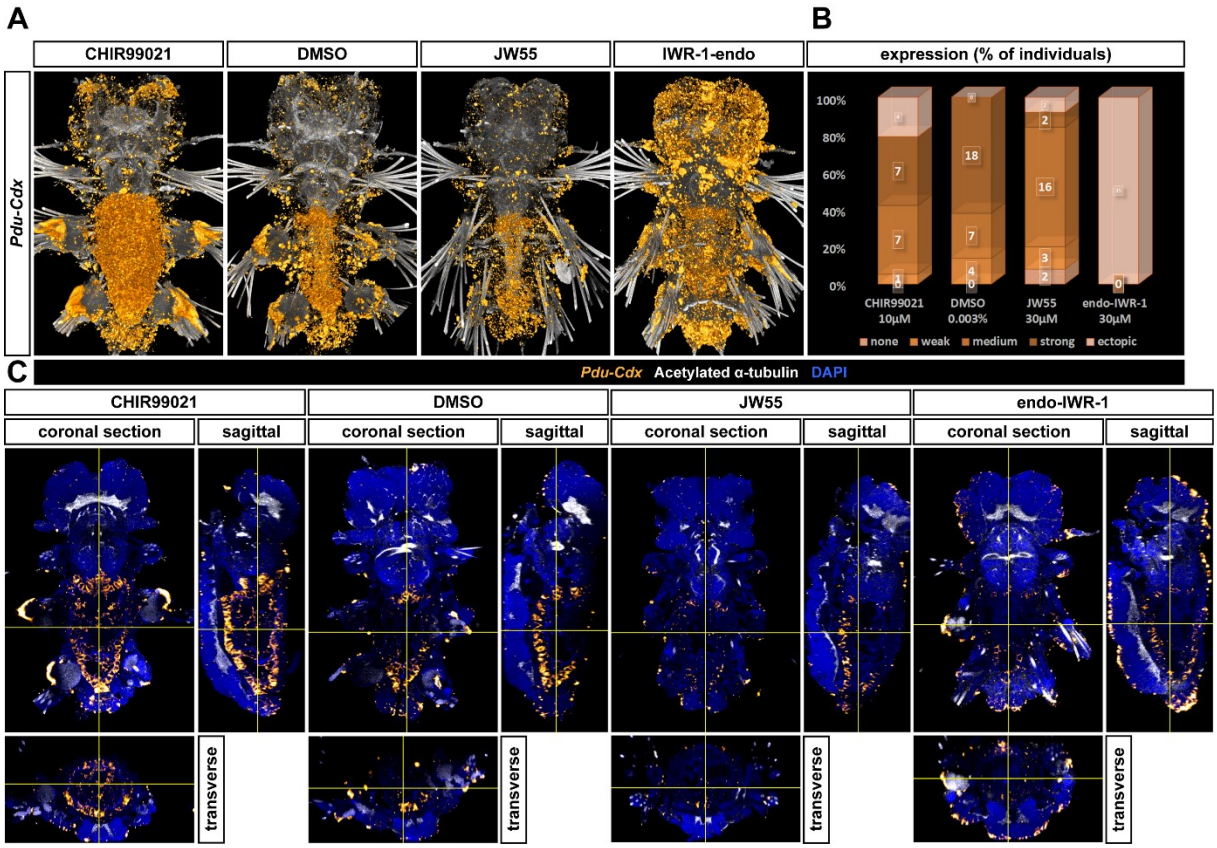

Figure 28 – The expression of the *ParaHox* gene *Pdu-Cdx*

(A) The dorsal views of 3D reconstructed confocal *z*-stacks of fluorescent *in situ* hybridization with a probe recognizing mRNA of a *ParaHox* gene *Pdu-Cdx* on 7 dpf *Platynereis* larvae treated from 5 to 7 days of development with chemical modulators of Wnt/β-catenin signalling. The images are oriented with their anterior facing to the top. In normal conditions (DMSO control), *Pdu-Cdx* is expressed in the whole hindgut, on the ventral floor of midgut (especially in the midline) and on the foregut/midgut border. Upon Wnt/β-catenin activation by CHIR99021, it is expressed in the entire gut, whereas with the inhibitor JW55, the expression is somewhat lower but the pattern remains the same. Ectopic expression with IWR-1-endo is obscuring the view but the gut expression seems to be similar to those in JW55 treated animals.
(B) Quantification of the effect of Wnt/β-catenin pharmacological treatment by the percentage of individuals in each phenotypic class to which they have been assigned according to their level of *Pdu-Cdx* expression (the darker the higher). A widespread ectopic expression with the stronger inhibitor (IWR-1-endo) is making proper quantification by the assignment of larvae to the groups according to their gut expression impossible.
(C) Orthogonal sections of the same *z*-stacks as in (A) overcome the problem of superficial signal obscuring the view on the gut. They reveal the expression in the hindgut and at the midgut/foregut boundary connected by a narrow strip of *Pdu-Cdx* expression in the ventral midline of the gut wall.

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(Figure 28 – continuation from the previous page)

This expression expands to whole ventral half of the (and - in somewhat lower level – to the entire) midgut upon Wnt activation. The expression of *Pdu-Cdx* drops down after Wnt/β-catenin inhibition, especially in the regions where it is normally present in high levels (the hindgut, the midgut/foregut boundary) and especially with the stronger inhibitor IWR-1-endo.
Coronal sections are oriented with the anterior of larva oriented to the top, *sagittal* sections with ventral to the left and the *transversal* sections are facing ventral down.

The inhibition of the Wnt/β-catenin pathway by either JW55 or IWR-1-endo causes *Pdu-Cdx* to be reduced or to almost disappear from the gut (Figure 28A, B – the third and fourth panels of images from the left). It persists the most in the ventral gut midline, closest to the strong Wnt source in the ectodermal ventral midline.

The observed shifts in *Pdu-Cdx* expression upon the manipulation with the activity of Wnt/β-catenin pathway does support it as a Wnt target gene (direct or indirect). However, despite Pdu-Cdx still might be involved in the initial specification of the identity of gut compartments according to their position along the A-P axis, it is apparently not involved their functional differentiation and hence in the midgut-to-hindgut conversion induced by an inhibition of Wnt/β-catenin signalling.

10.4.4. Proliferation, cell death and survival

Wnt/β-catenin is crucial for the maintenance of stem cells and hence for the generation of proliferating progenitors in many tissue, e. g. the central nervous system (section 7.2.3.2) or the intestine (section 7.2.4.3). It was thus possible that the observed changes in gene expression after the manipulations of Wnt/β-catenin signalling were a consequence of a deregulated balance between the proliferation, differentiation and cell death. To evaluate this hypothesis, I investigated the cell proliferation and death in control, mock-treated 7 dpf *Platynereis* nectochaete and the larvae treated during the nectochaete stage (from 5 to 7 dpf) by activator (CHIR99021) or inhibitor (IWR-1-endo) of Wnt/β-catenin signalling.

10.4.4.1. Cell proliferation

To evaluate cell proliferation, I added a solution of 5-ethynyl-2'-deoxyuridine (EdU) at 6 dpf to the larvae developing from the 5 dpf in the presence of pharmacological modulators of Wnt/β-catenin signalling or the mock DMSO treatment (section 9.10.1.1 of Materials and methods). I then imaged multiple larvae by a spinning disc confocal microscopy and counted manually the positive dividing cells on maximal projections of confocal *z*-stacks.

The highest number of dividing cells is found in the growth zone between the pygidium and the last segment, around the pharynx, bases of parapodia, cirri and in the anterior of the head (Figure 29A). The number of dividing cells remains on average more or less the same after the activation of canonical Wnt signalling but is significantly and almost completely abolished in the entire body of the larva if the Wnt/β-catenin signalling is inhibited (Figure 29B). This result demonstrates that Wnt/β-catenin signalling is necessary for cell proliferation in the larvae of *Platynereis* in general.

10.4.4.2. Cell death

There still remained the possibility that the changes in gene expression could be caused by cell death caused by the used pharmacological inhibitors. To check whether the inhibition of Wnt/β-catenin signalling and the drop in cell proliferation are not connected with an elevated cell death, I took advantage of the TUNEL technique that marks the ends of fragmented DNA in dying cells with a fluorescent label and used it to evaluate cell death in 7 days old *Platynereis* control larvae or larvae of same age after pharmacological treatment with modulators of Wnt/β-catenin pathway (Figure 29C).

I found that there were more dying cells when either the activator CHIR99021 or the inhibitor IWR-1-endo of Wnt/β-catenin signalling were used, but not dramatically (Figure 29D). More importantly, the level of cell death was similar with either compound, in contrast to the opposite effects of activation and inhibition on gene expression in the gut. This is a different situation from the neuroectoderm, where either activation or inhibition often yielded similar results in gene expression. A similar level of cell death after either treatment also means that the cell death also did not depend on the Wnt/β-catenin signalling. Subjectively, the cell death seemed to be more advanced in the IWR-1-endo-treated larvae but on the surface and not in the gut.

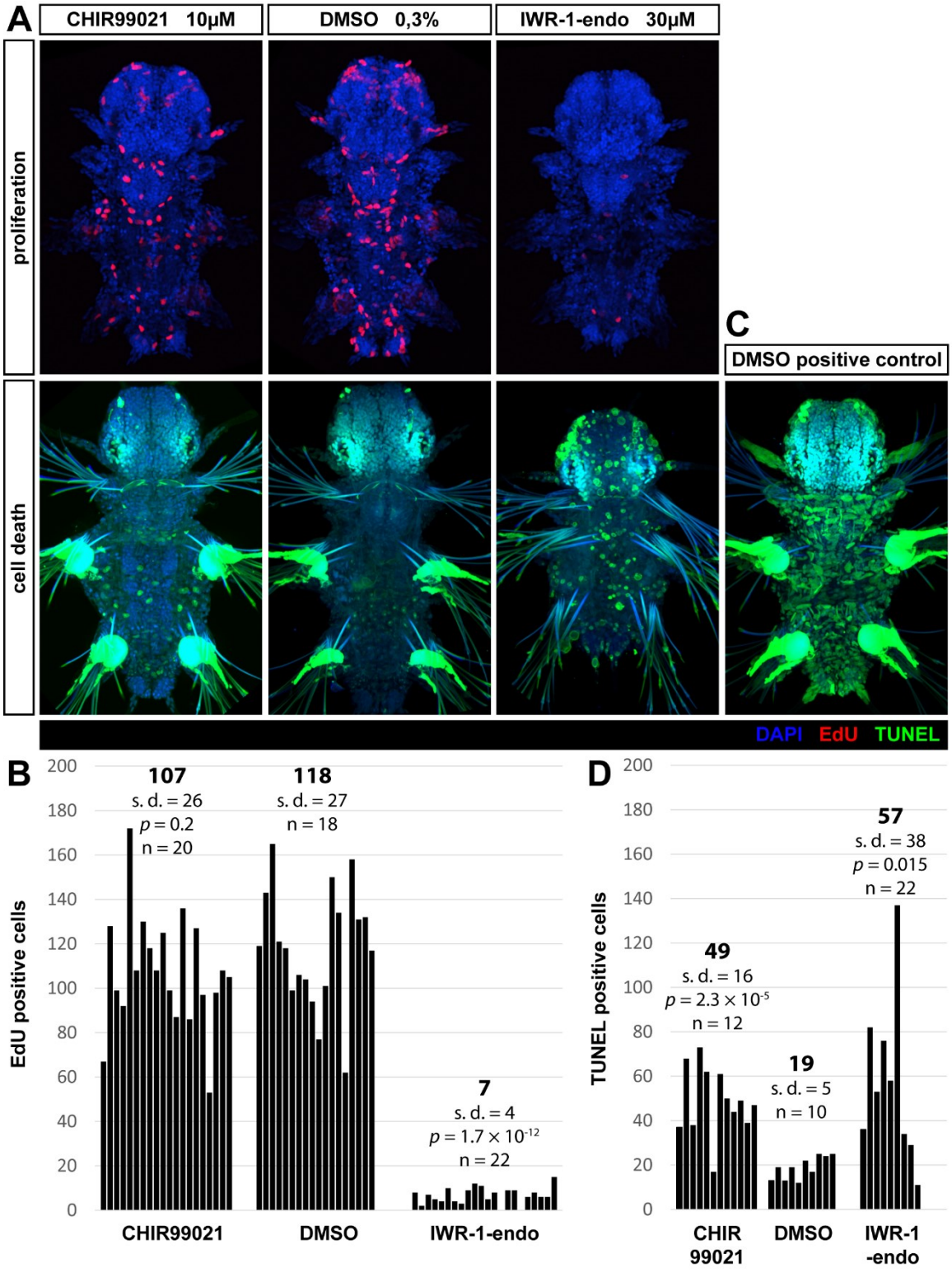

Figure 29 – Wnt/β-catenin signalling is necessary for cell proliferation and survival

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Figure 29 – Wnt/β-catenin signalling is necessary for cell proliferation and survival

(A) Maximum *z*-projections of fluorescent confocal *z*-stacks of 7 dpf *Platynereis* larvae treated with Wnt/β-catenin modulators from 5 to 7 dpf. The proliferating cells were labelled by incorporation of 5-ethynyl-2'-deoxyuridine (EdU, *red*) added to the water with developing larvae from 6 to 7 dpf and after fixed, cell nuclei were counterstained with DAPI (*blue*). Representative individuals are shown.
(B) Number of EdU positive proliferating cells in the entire body of individual larvae as counted manually on maximal projections of *z*-stacks as those shown in (A). There is no significant difference in the number of proliferating cells upon the activation of Wnt/β-catenin signalling by CHIR99021 but there are significantly less proliferating cells in the presence of the Wnt/β-catenin inhibitor IWR-1-endo.
Averages are indicated in bold, *n* – number of individuals analysed per each experimental group, *p* – p-value of a standard two tailed unpaired Student's t-test with unequal variance, *s.d.* – standard deviation.
(C) TUNEL staining of nicked/fragment DNA ends (*green*) to mark the cell death in 7 dpf *Platynereis* larvae after pharmacological treatment with Wnt/β-catenin modulators from 5 to 7 dpf and counter-stained with Hoechst dye (*blue*) after fixation to visualize nuclei.
(D) Quantification of cell death performed in the same way as for proliferation except that this time, only cells from the trunk up to midgut/pharynx boundary (or up to the first pair of parapodia) were counted. There are significantly more dead or dying cells in groups treated with either CHIR99021 or IWR-1-endo compared to control group treated with DMSO alone.
The images are maximum projections of fluorescent confocal *z*-stacks oriented with their ventral to the top.

10.4.4.3. Endogenous alkaline phosphatase

Endogenous alkaline phosphatase is an enzyme that is naturally present in the epithelia of the digestive tube and nephridia in *Platynereis* (Hasse et al., 2010). It could thus serve to determine which and how many of the dividing cells are located to the gut. I thus used *in situ* hybridization substrate and simplified procedure in combination with EdU labelling to detect dividing cells in the midgut.

The pattern *Pdu-AP* is not changed by either the activation or inhibition of Wnt/β-catenin signalling (Figure 30). I found that only few dividing cells were located to the midgut and most of the growth of the gut probably takes place in the region around the hindgut and on the midgut/foregut border.

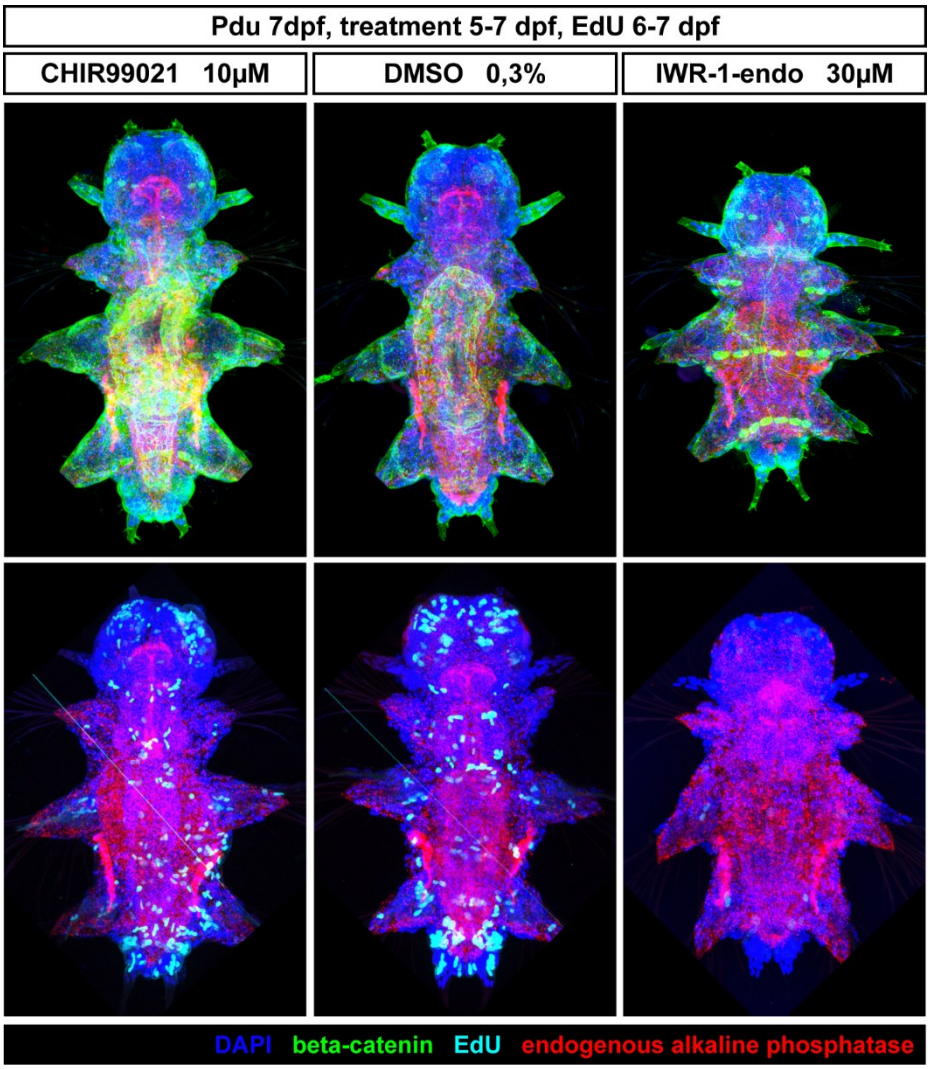

Figure 30 – The activity of endogenous alkaline phosphatase

β-catenin and cell proliferation in a relation to a digestive and excretory system, which has been visualized by the fluorescent *in situ* hybridization substrate catalysed by the endogenous alkaline phosphatase.

In control larvae (DMSO), β-catenin can be found in the midgut as described before, but not in the foregut, the hindgut or nephridia, where the endogenous alkaline phosphatase is also present. β-catenin overall level (including the gut) is elevated after the activation of Wnt/β-catenin pathway by CHIR99021, while it almost completely disappears from the gut (and is lowered overall) when the Wnt/β-catenin signalling is inhibited by IWR-1-endo.

Co-staining of endogenous alkaline phosphatase with EdU detection of proliferating cells shows, that there are not many dividing cells in the midgut. Instead, the proliferative activity can be seen in the foregut, the foregut/midgut and midgut/hindgut borders, the hindgut and the ring of cells around the hindgut (the segment addition zone, SAZ), the pygidium, parapodia and in the head lobes. Only few positive proliferating cells can be found on the ventral side in the middle of the midgut. These cells might correspond to primordial germ cells (PGCs).

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(Figure 30 – continuation from the previous page)

The activation of Wnt/β-catenin signalling by CHIR99021 has no pronounced effect on cell proliferation. Even though here is shown an individual with slightly higher number of proliferating cells in the gut, this effect is not significant if averaged. In contrast, the inhibition of the pathway by IWR-1-endo abrupts the cell proliferation completely throughout the body.
The images are maximum projections of fluorescent confocal *z*-stacks of 7 dpf *P. dumerilii* larvae treated with pharmacological modulators of Wnt/β-catenin signalling from 5 to 7 days of development.

10.4.4.4. Survival and feeding

I was then curious whether the observed misdifferentiation of gut compartments and aborted proliferation (for inhibition) and/or increased apoptosis (for either activation or inhibition) represent a permanent event that would compromise the further survival of the larvae. For this purpose, I alleviated the action of pharmacological modulators of Wnt/β-catenin signalling that was imposed on larvae from 5 dpf to 7 dpf by moving the larvae to a clean natural sea water. I then continued to cultivate them for the following two weeks in order to see if they survive, grow and feed.

I observed that more than 60 % larvae in all experimental groups survived, out of which 85 – 92 % had their guts filled with algae by the day 21 of development (Figure 31). They also apparently continued to grow. After I let the larvae to starve for two more days in order to get their guts clean of the autofluorescent *Tetraselmis* algae which they were fed with, I also checked for the expression of some gut markers from the Figure 26. Larvae from all experimental groups showed expression patterns similar to those observed at 7 dpf or to the DMSO control at this (23 dpf) stage, but the number of larvae at the end of in situ protocol was too low and the variability too high because the development is already asynchronous at this stage. Therefore, it was not possible to properly score the results of *in situ* hybridization in a way similar to how it was achieved for 7 dpf larvae.

I found out that the larvae can overcome changes caused by the pharmacological activation or inhibition of Wnt/β-catenin pathway from 5 to 7 dpf, survive, feed and grow. The observed misdifferentiation of gut compartments caused by inhibition of Wnt/β-catenin is thus not permanent and the cells of digestive epithelium can assume proper fate once the canonical Wnt signalling in the gut is reconstituted.

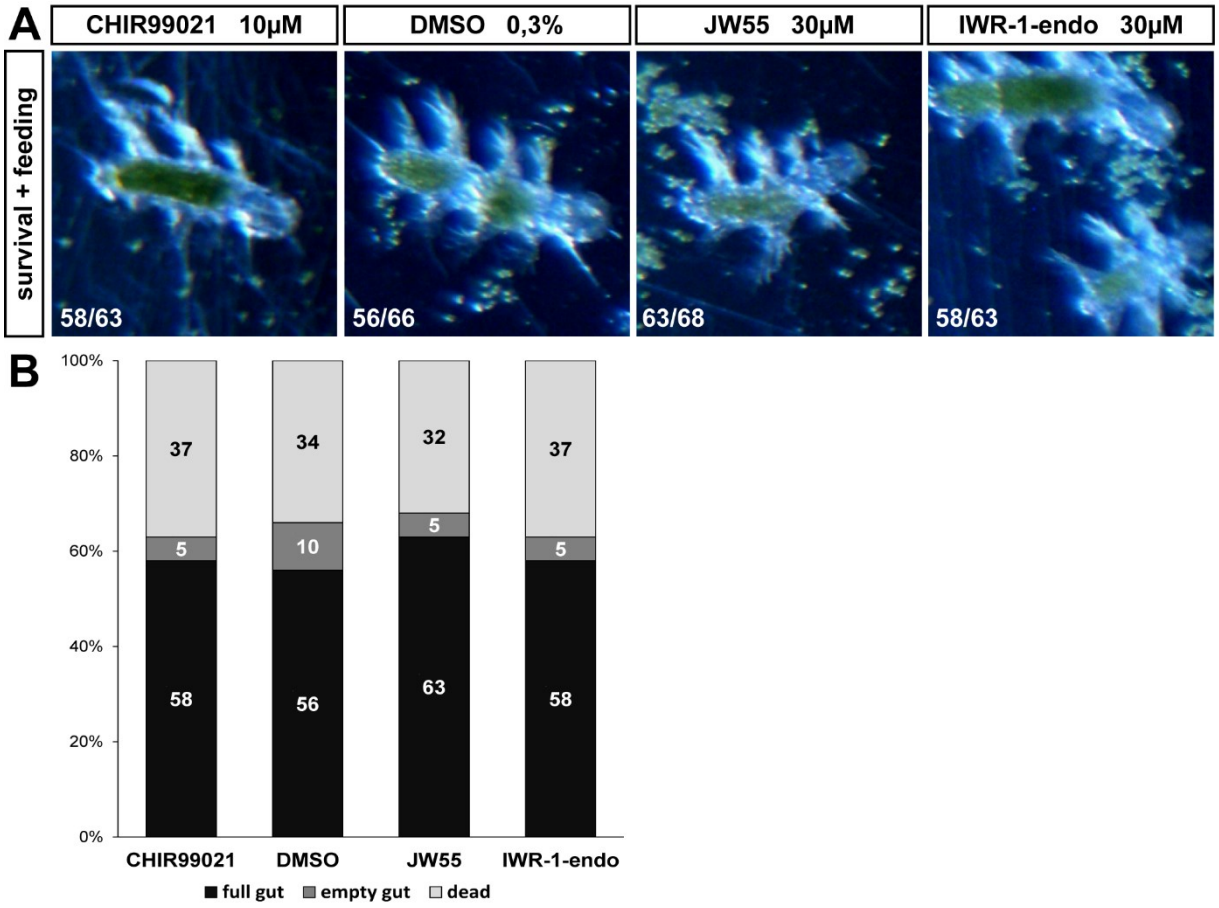

Figure 31 – Larvae can overcome a period of Wnt manipulation and continue to feed and survive

(A) Even after a treatment with Wnt activator or inhibitors between the day 5 and 7 of development, the larvae are able to recover. 21 dpf stage fed from 7 dpf by *Tetraselmis* unicellular benthic algae is shown here. The numbers indicate number of feeding juvenile worms over the number of surviving individuals from the original one hundred after the end of treatment in each experimental group.
(B) Different graphical representation of the data from (A) – a quantification of the larval survival and feeding at 21 dpf after the manipulation of Wnt/β-catenin pathway between the day 5 and 7 of development. 100 larvae were included in each experimental group by the end of pharmacological treatment at 7 dpf. The number of surviving larvae is similar in each group and varies in the range of 63-68 %. Also the percentage of the feeding larvae is approximately the same in each group in the range from 85 to 92 %.
Only one replicate was done and hence no statistics is shown.

10.4.5. Addendum: Muscle development

The gut mesoderm has an important role in the differentiation of gut epithelium in both vertebrates and insects (section 7.2.4.3). It was possible that the manipulation with the activity of Wnt/β-catenin signalling affected also the smooth musculature of the gut compartments. To test this and find out whether the muscles could be involved in the Wnt/β-catenin signalling in the gut, I used fluorescently labelled phalloidin to detect actin filaments in muscle cells (Figure 32).

The staining in the control 7 dpf larva which was treated only with DMSO from 5 dpf revealed all major striated muscles as they were described at about the same time for *Platynereis* nectochaete (Brunet et al., 2016). Wnt/β-catenin signalling thus did not affect differentiation of striated muscles at this stage which is not surprising given that it takes place already during the metatrochophore stage (Fischer et al., 2010). However, just few (almost none) smooth muscle fibres are present at that time in the walls of the midgut and hindgut because the muscles of the gut wall just started to differentiate except for the pharynx in which the differentiation of muscle fibres starts earlier and can be already seen at 7 dpf. All striated muscles are present even after either activation or inhibition of Wnt/β-catenin signalling. They are shorter and the musculature is consequently more compact in the larvae in which the canonical Wnt signalling was inhibited by IWR-1-endo and reminds the musculature of younger stages (Fischer et al., 2010). This corresponds well to the observed overall inhibition of proliferation (and hence implicated arrested growth) of the IWR-1-endo-treated larvae (10.4.4.1).

Nevertheless, it is still cannot be excluded that the Wnt signalling in the gut involves a non-muscle mesoderm and the misdifferentiation of the digestive epithelium could be just a secondary cause of a misregulated development of the gut mesoderm.

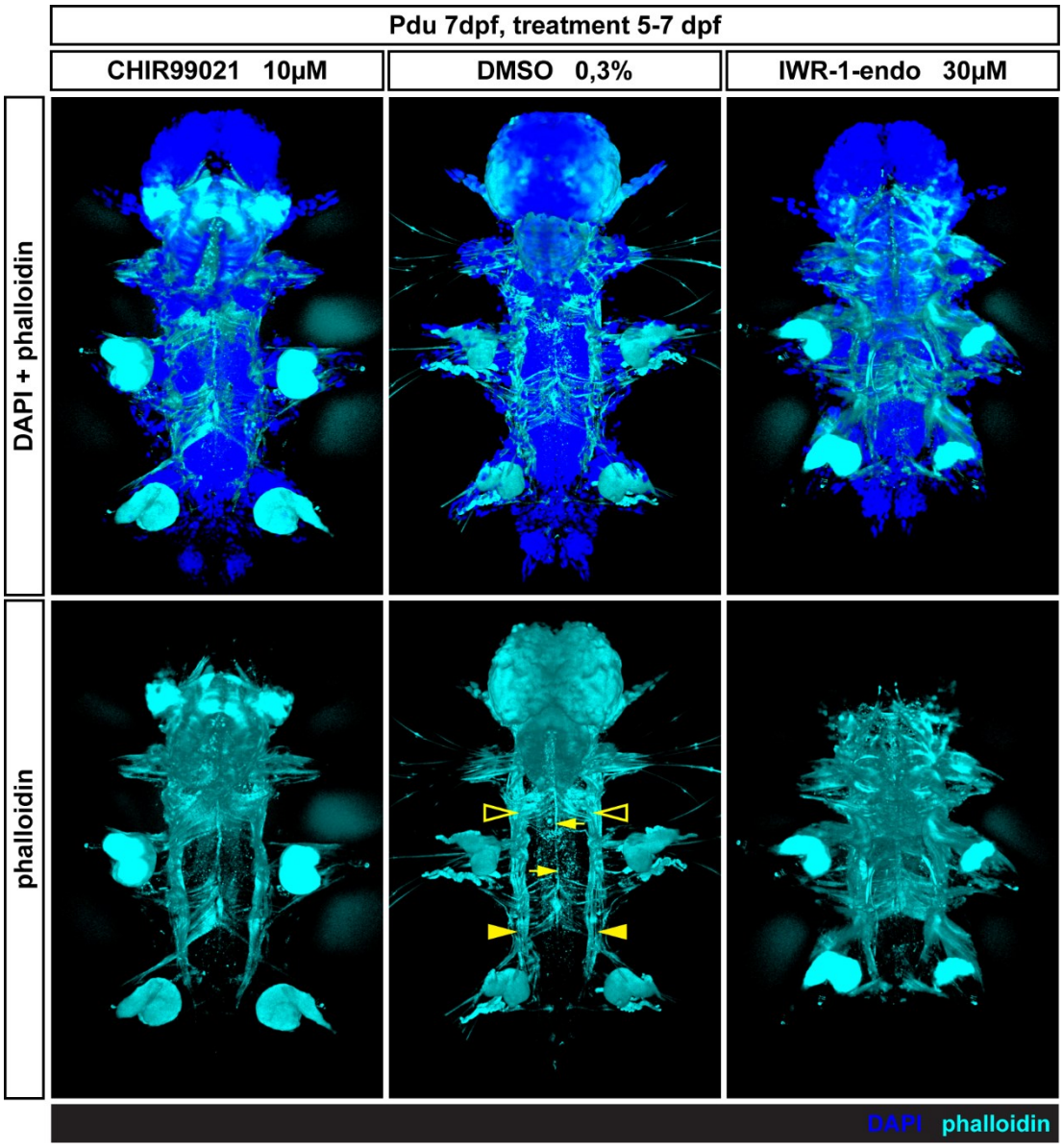

Figure 32 – Phalloidin staining of muscles after Wnt treatment

Dorsal views of 3D projections of 7 dpf *Platynereis* larvae after pharmacological treatment from 5 to 7 dpf with actin stained by fluorescently labelled phalloidin. It is possible to observe major body musculature, i. e. ventral longitudinal muscles for lateral bending during crawling, ventral oblique muscles and parapodial muscles to control the movements of parapodia and the ventromedian axochord, a muscle, which has been homologized to the vertebrate notochord (Brunet et al., 2015; Lauri et al., 2014), but no staining of actin in smooth muscles in the gut wall.

All observed muscles are present after either activation or inhibition of Wnt/β-catenin signalling. The signal in the head and spinning glands of parapodia is unspecific background.

top row – phalloidin labelling + nuclei counterstained with DAPI; *bottom row* – phalloidin only. The larvae are oriented with their anterior to the top.

empty arrowheads – ventral oblique muscles, *filled arrowheads* – ventral longitudinal muscles, *arrows* – ventromedian muscle (axochord).

11 Discussion

11.1. Wnt/β-catenin pathway in *Platynereis*

11.1.1.1. Wnt proteins activate the Wnt/β-catenin pathway in the neighbouring tissue

I investigated the expression of several components of the Wnt/β-catenin pathway in order to reveal the sites of its activity. The expression patterns for *Pdu-Wnt* genes were already known (Pruitt et al., 2014) and due to a prevailing paracrine short-range signalling of Wnt proteins (Clevers and Nusse, 2012) thus should in theory well reflect the sites of the activity of the Wnt/β-catenin signalling. The expression of *Pdu-Wnt* genes can be found in the ventral midline, segmental stripes, the pygidium and the lateral episphere.

Nevertheless, I also tracked the expression of *Platynereis* homologue of a member of the β-catenin destruction complex *Pdu-Axin*. *Axin2*, but not *Axin1* is also the Wnt target gene in mammals and hence is upregulated in the signal receiving cells instead of the Wnt secreting cells and can serve as a read-out of the Wnt/β-catenin pathway's activity (Jho et al., 2002; Lustig et al., 2002). Since *Platynereis* has only one *Axin* orthologue, it was not clear from the beginning whether it is a target of the Wnt/β-catenin pathway or not. This was confirmed by an upregulation of *Pdu-Axin* expression in response to the pharmacological activation of the Wnt/β-catenin signalling and a downregulation upon the pathway inhibition from 24 to 48 hpf (Figure 6). Interestingly, *Pdu-Axin* is mRNA is detected among others along the Wnt-secreting ventral midline at 48 hpf as was already published before (Demilly et al., 2013) and in the putative segment addition zone (SAZ) between the pygidium and the last body segment in the nectochaete larvae on the day 7 of development (Figure 7A). If we compare *Pdu-Axin* expression with the expression of *Pdu-Wnt* genes, we get to the interesting conclusion that the Wnt/β-catenin signalling is triggered in cells neighbouring the signal producing cells but not in the Wnt-secreting cells themselves.

11.1.1.2. Is Wnt/β-catenin signalling active in the gut?

The Wnt/β-catenin signalling can influence the transcription only in the presence of its effectors. Therefore, I isolated the *Platynereis* orthologue of the endpoint effector transcription factor of the Wnt/β-catenin pathway *Pdu-Tcf*. Its developmental expression

was in a good congruence with the published patterns of *Pdu-Wnt* genes and with the expression of *Pdu-Axin*. Nevertheless, the expression of *Pdu-Tcf* in the midgut of 7 dpf nectochaete larvae was novel as the Wnt/β-catenin has never been studied previously at this stage. It suggested that the Wnt/β-catenin signalling might be active there. However, some Tcf proteins are known to behave as activators of transcription of target genes in the presence of a Wnt signal whereas they act as repressor in the absence of a signal. Others can behave as repressors even in the presence of the Wnt signal (see the section 7.1.4.4). Therefore, a mere presence of this transcription factor did not necessarily mean the activation of Wnt target genes. The BLAST search returned Tcf7l2 (Tcf4) as top hit from most organisms with more Tcf orthologues (Figure 10A). Interestingly, Tcf7l2 is the vertebrate Tcf paralogue that can have both activating and repressive function (see the section 30). It is tempting to speculate that the similarity in sequence might reflect the similar properties of Pdu-Tcf and vertebrate Tcf7l2 and is capable of either activation or inhibition. However, despite Pdu-Tcf contains all typical domains of Tcf proteins (Figure 10B), including the Groucho binding sequence that confers the inhibitory function in the absence of the signal (7.1.4.1, 7.1.4.4), it lacks the amino acid signature which is characteristic for the inhibitory isoforms of Tcf3 and Tcf4 (section 10.1.5.1) and it should thus act as an activator of transcription in a presence of the Wnt signal.

Also the expression of other member of the Wnt/β-catenin pathway *Pdu-Axin* was confined to the gut. Because the pharmacological treatments with the activators and inhibitors of Wnt/β-catenin signalling pathway confirmed that it is a target of Wnt/β-catenin signalling, the expression in the midgut of 7 dpf larvae probably results from the active Wnt/β-catenin signalling in this tissue (Figure 7A). Interestingly, the effect of Wnt/β-catenin pathway inhibition from 5 to 7 dpf on the *Pdu-Axin* expression was relatively mild and the activation of the canonical Wnt pathway was not able to elevate *Pdu-Axin* levels in the gut or anywhere else in the body (Figure 7A, B). This raised the possibility that there is an efficient homeostatic mechanism in place at this stage, which is able to buffer the overactivation of the Wnt/β-catenin signalling and restrict its activity to certain tissues. I argue that this was most probably the reason for the absence of any phenotype in the gut expression after the activation of the Wnt/β-catenin signalling from 5 dpf to 7 dpf.

The hypothesis that the Wnt/β-catenin pathway is active in the midgut was further supported by elevated levels of β-catenin proteins observed in this compartment (Figure 7C). Although I was not able to recognize its nuclear localization from the cytoplasmic and cytoskeletal fraction, either the signalling and the structural function share a common pool

of β-catenin (Hülsken et al., 1994). Even the total amount of β-catenin protein thus indicates the activity of the Wnt/β-catenin pathway in the midgut.

As a final proof of the canonical Wnt pathway activity in the midgut compartment, I injected a Wnt-responsive construct with multiple Tcf/Lef binding sites into the *Platynereis* zygotes. However, the initially intended destabilized GFP was to weak and I had to switch to a stable but bright tdTomato. Despite tdTomato is very stable and could potentially remain in the cell a long time after it has been synthesized, its fluorescence in the midgut of 7 dpf larvae quite conclusively proved that the Wnt/β-catenin signalling is active during the gut development.

11.1.1.3. Pdu-Tcf exists as multiple isoforms

The first *Pdu-Tcf* which I have isolated lacked a C-terminally located accessory DNA binding domain called C-clamp. This domain is present in all invertebrate Tcf proteins known from organisms with a single Tcf paralogue (Archbold et al., 2012; Cadigan and Waterman, 2012). I was thus curious whether *Platynereis* has more *Tcf* genes or if becomes the first known organism without a Tcf with the C-clamp known to date. Both these options could be soon rejected because it turned out that these fragments represent different isoforms of a single gene. They contain alternative exons for the DNA binding HMG domain that can be freely combined with C-clamp domains of three different lengths generated from the same exon by an alternative splicing. Alternatively, the C-terminal part could be omitted completely, which yielded in total eight *Pdu-Tcf* mRNA variants.

It might be presumed that these variants would differ in their DNA binding capacity and hence in their selected target sites, developmental function and expression. They could also differ in their activation properties as the C-clamp containing E-tail binds CtBP; however, CtBP can be either co-activator (Valenta et al., 2003) or a co-repressor (Brannon et al., 1999) of transcription, depending on the context. I tried to find out which *Pdu-Tcf* variants are expressed in the gut and surprisingly, out of the six tested isoforms, all displayed the same expression pattern and none of them was detected in the gut.

Although the gut-specific variant has not been determined yet, *Pdu-Tcf* was detected in the midgut by a universal N-terminal probe, which means that at least some isoforms have tissue-specific expression. Tissues might differ in the ratio of Tcf isoforms which they produce and the alternative splicing increase the plurality of responses to the Wnt/β-catenin pathway in the protostome lineage to compensate for the lack of Tcf paralogues.

11.2. Neuroectoderm

11.2.1. Anterior-posterior patterning

11.2.1.1. Wnt/β-catenin does not affect the A-P patterning of neuroectoderm in the Platynereis trochophore

Quite unexpectedly, the pharmacological treatments of the Wnt/β-catenin signalling pathway led to a decreased expression but did not shift anterior-posterior boundaries of the expression of the transcription factors *Pdu-Otx* (Figure 21), *Pdu-Hox*, *Pdu-Hox4* (Figure 23,) and *Pdu-Lox* that confer the anterior-posterior identity of the developing CNS.

The posterior border of another A-P patterning TF *Pdu-Six3* retracted slightly towards the anterior in the episphere; however, this anterior shift was observed after either the activation or inhibition of the Wnt/β-catenin pathway. *Pdu-Six3* is the gene with the anterior-most expression so that the anterior shift of its posterior boundary in the episphere does not have to necessarily represent a true shift of the entire *Pdu-Six3*-expression domain but instead a reduction of its size. The reason that could cause such reduction will be discussed in the chapter 11.2.2.

Pdu-Six3 was also expressed ectopically in the hyposphere after the activation of Wnt/β-catenin pathway and decreased in the episphere after the inhibition (Figure 21) – the opposite of what would be expected from the anterior marker gene that is negatively regulated by a posterior Wnt signals in vertebrates (Braun et al., 2003). The ectopic expression of *Pdu-Six3* in the hyposphere could be a result of an unspecific upregulation of neural-related genes due to the overactivation of canonical Wnt signalling. But there is also another, more elegant solution: Six3 in known to function as a repressor of Wnt/β-catenin signalling during the development of telencephalon in vertebrates (Lagutin et al., 2003). It is possible that *Pdu-Six3* itself is positively regulated by the canonical Wnt pathway and engages in a negative feedback loop that buffers the Wnt/β-catenin in the neuroectoderm.

The anterior-posterior patterning of the neuroectoderm thus seems not to be regulated by Wnt/β-catenin signalling in *Platynereis* trochophore from 24 to 48 hpf, which was quite unexpected given that Wnt/β-catenin signalling is involved in the A-P patterning of the CNS in most other bilaterian animals (section 7.2.3.3). However, it is possible that the Wnt/β-catenin signalling is necessary only to establish the expression of the A-P neural patterning TFs, which then become restricted to their respective expression domains by cross-regulation and/or they are limited to these boundaries by other signalling pathways.

This hypothesis however does not explain the observed decrease in expression and/or the reduction of domain sizes upon either the activation or inhibition of the Wnt/β-catenin signalling. There is an explanation for this phenomenon but to identify it, we have to investigate also the medio-lateral patterning of *Platynereis* CNS(chapter 11.2.2).

Nevertheless, even if the A-P patterning did not provide much information about the role of Wnt/β-catenin signalling in the *Platynereis* CNS development, it is still possible to explore the obtained wild type (DMSO control) gene expression data. Together with a re-examination of previously published gene expression patterns, it might be possible to use them to identify homologues of vertebrate CNS signalling and tissue organizing centres in the developing *Platynereis* neuroectoderm and to homologize its regions with compartments of the vertebrate brain.

11.2.1.2. Is there an isthmic organizer in Platynereis?

Putative homologues of the vertebrate isthmic organizer (IsO, located on the midbrain-hindbrain boundary, MHB; section 7.2.3.3) were found in the collar of hemichordate larvae (Pani et al., 2012) and between the deuterocerebrum and tritocerebrum of *Drosophila* (Hirth et al., 2003). However, the conservation of the IsO gene expression fingerprint is not absolute and displays some differences (Urbach, 2007). A homologue of IsO has never been documented in the third large bilaterian phylogenetic group, the Spiralia, and its presence in the last common ancestor of Protostomia and Deuterostomia (i. e. all Bilateria, hence urbilaterian ancestor) thus remains uncertain.

The isthmic organizer of vertebrates forms at the boundary between the anterior *Otx* and posterior *Gbx* positive regions, where the expression of *Wnt, FGF*, *Engrailed* and *Pax2/5/8* is being established (see the section 7.2.3.3). Although it was not a primary goal to study the isthmic organizer in *Platynereis*, I detected for other purposes by *in situ* hybridization the expression of IsO-specific genes *Pdu-Otx*, *Pdu-Engrailed*, *Pdu-Pax2/5/8* and of three *Pdu-Wnt* genes (although not of *Pdu-Wnt1* and *Pdu-Wnt8* whose orthologues are involved in the formation of MHB in vertebrates). Wild type expression patterns of all these IsO-specific genes except for *Pdu-FGF* were already described in the literature (Arendt et al., 2001; Denes et al., 2007; Dray et al., 2010; Prud'homme et al., 2003; Pruitt et al., 2014; Steinmetz et al., 2011). However, they were not inspected with respect to a possible conservation and an urbilaterian ancestry of the isthmic organizer. If I compare the published expression patterns with my high-resolution fluorescent *in situ* hybridization

stainings on wild type (i. e. DMSO control) larvae, it might allow me to formulate a hypothesis about the homology of IsO signalling centre and hence the midbrain and hindbrain in *Platynereis*.

It has been described for *Platynereis* trochophore that *Pdu-Gbx* is expressed in the "zero" cryptic segment on both sides of the stomodaeum between the peristomium and the first chaetigerous segment (Steinmetz et al., 2011). *Pdu-Otx* is can be found in the peristomium (Arendt et al., 2001) despite my data suggest that its expression continues to the ventral nerve cords at the early metatrochophore stage (48 hpf) but becomes to be confined to the episphere and the peristomium before a larva reaches the late metatrochophore stage (72 hpf; Figure 21). Hence it looks like the peristomium-zero segment boundary could fulfil the condition for the IsO which is a sharp border between the anterior *Otx* and posterior *Gbx* expression due to their mutual repression (Broccoli et al., 1999; Katahira et al., 2000; Martinez-Barbera et al., 2001; Millet et al., 1999). Also the widespread expression of *Pdu-Pax2/5/8* in the hyposphere overlaps the boundary between the zero segment and the prostomium at the level of the VNCs [Figure 20 and (Denes et al., 2007)]. The segmentally expressed *Pdu-En* can be found in either the cryptic zero segment and the peristomium and crosses their boundary laterally to the stomodaeum [the expression in the peristomium and cryptic segment can be seen in the Figure 24, a crossing of the boundary is best apparent on the *in situ* hybridization published by Dray et al. (2010)]. My data are not sufficient for an exact evaluation, but the expression of MHB-related genes *Pdu-Pax2/5/8* (Figure 20) and *Pdu-En* (Figure 24) seems to be downregulated in the critical region of VNCs on the peristomium/zero segment boundary as a part of general downregulation upon an inhibition of Wnt/β-catenin pathway.

The expression of *Pdu-Wnt10*, whose orthologue acts redundantly with Wnt1 in the zebrafish MHB (Lekven et al., 2003), reaches almost to the middle of the stomodaeum and is expressed just posterior to the prototroch, probably in the peristomium (Pruitt et al., 2014), consistent with the role of Wnt10 in the ventral MHB (ventral in vertebrates is medial in *Platynereis*); *Pdu-Wnt1* is expressed more laterally but it is possible that it can also reach to the stomodaeum (Dray et al., 2010). Also *Pdu-Wnt8*, the orthologue of *Wnt8* which is involved in the positioning of isthmic organizer in vertebrates (Rhinn et al., 2005), and several other *Pdu-Wnt* genes like *Pdu-WntA* and *Pdu-Wnt16* that were investigated here (Figure 5) are expressed lateral to the stomodaeum on the peristomium-trunk boundary (Pruitt et al., 2014). Wnt3, the last Wnt known to function in the MHB of vertebrates, is

present only in Deuterostomia and Cnidaria but is absent from all Protostomia (Holstein, 2012).

The region lateral to the stomodaeum on the peristomium-trunk boundary also exhibits a gap in the expression of *Pdu-Nk2.2* (Denes et al., 2007). An analogous gap can be found on the midbrain-hindbrain and deuterocerbral-tritocerabral boundaries of vertebrates and insects, respectively (Urbach, 2007).

Taken together, the expression data suggest that a homologue of the isthmic organizer (midbrain-hindbrain boundary) is present in *Platynereis dumerilii*. It is positioned on the border of the peristomium and the cryptic zero segment which also becomes a part of the head. This in fact represents a boundary between the non-gastrulating ectoderm of the episphere and prostomium and the ectoderm of the trunk which gastrulates by epiboly (see the section 7.3.5.2).

11.2.1.3. The assessment of homology between the trochophore neuroectoderm and the vertebrate brain

The proposed position of the IsO homologue would implicate that parts of the CNS within the peristomium is homologous to (at least) the midbrain of vertebrates whereas the part of the VNCs posterior to the peristomium-zero segment boundary would correspond to the hindbrain. Indeed, two ventral-lateral cells cMN^{cl} and cMN^{ATO} in the prostomium region express tyrosine hydroxylase, a marker of dopaminergic neurons (Verasztó et al., 2017). They are most probably identical to the *cp1*, *2* and *cv1*, *2* catecholaminergic neurons described by others Starunov et al. (2017). Dopaminergic neurons are characteristic for midbrain where their proliferation and differentiation are regulated by Wnt/β-catenin signals from the isthmic organizer (Castelo-Branco et al., 2010; Castelo-Branco et al., 2004; Castelo-Branco et al., 2003). Moreover, the regions just posterior to the boundary, at the sites of connection of the first ventral commissure to the VNCs contain serotonergic cells (Fischer et al., 2010). Serotonergic cells are typically found in the caudal linear nucleus of the metencephalic (hindbrain) raphé where they function as central pH chemoreceptors (Severson et al., 2003).

If we consider a marked similarity between the molecular signature of the proposed homologue of IsO and the repetitive segmental pattern of *Pdu-Otx*, *Pdu-En*, *Pdu-Wnt* genes and the ubiquitous *Pdu-Pax2/5/8* in the *Platynereis* hyposphere, most of the IsO gene expression fingerprint is repeated in each segment. Even the expression domain of *Pdu-*

Pax2/5/8 sends segmental protrusions towards the ventral midline already in the 48 hpf stage [apparent in the *in situ* hybridization published by Denes et al. (2007)] and becomes segmental later in development in juvenile worms (Backfisch et al., 2013). I can only speculate that these variations on the IsO theme are formed in response to periodic iterations of the trunk-prostomium boundary Wnt signals during the segmentation of initially undivided trunk by a mechanism described in the section 11.3.1.2. They differ in the use of particular posterior homeobox gene – it is the *Pdu-Gbx* in the zero segment, *Pdu-Hox1* in the first chaetigerous segment, *Pdu-Hox4* in the second segment and *Pdu-Lox5* in the third (Figure 23, Figure 25). The IsO remains unique due to its anterior border with the peristomium and the stomodaeum The segmental boundaries hence probably contain only a subset of IsO specific genes and display some, but not all of its characteristics. This view is supported by a corresponding segmental occurrence of serotonergic (Fischer et al., 2010) but not catecholaminergic neurons (Starunov et al., 2017) in *Platynereis* metatrochophores.

By an analogy with the isthmic organizer in *Platynereis*, a second boundary with organizer function that separates domains with different gene expression should be located on the anterior peristomium border, i. e. in the prototroch which separates the episphere from the hyposphere (section 7.3.3.1). This boundary could be homologous either to the forebrain-midbrain boundary or to the zona limitans intrathalamica (ZLI), another organizer of the vertebrate brain development located anterior to the isthmic organizer. ZLI homologue might be located in two oblique domains of a neuroectodermal expression of *FoxA* in the domains in the medial episphere just anterior to the stomodaeum of *Platynereis dumerilii* and *Alitta virens* (Kostyuchenko et al., 2018). The orthologue of *FoxA* is active in the ZLI of vertebrates (Britto et al., 2002) and hemichordates (Pani et al., 2012). ZLI of either vertebrates and hemichordates is characteristic most of all by the presence of *Hedgehog* expression. In *Platynereis*, the mediator of Hh signalling *Pdu-Gli* is expressed in the domain that is reminiscent of *Pdu-FoxA* expression in the episphere (Dray et al., 2010). However, the available data does not allow me to assess whether the putative ZLI homologue is derived from the peristomium boundary or more anterior cells. In contrast, the boundary between *Pdu-Irx* and *Pdu-Fezf* (section 7.2.3.3) is located laterally in the episphere with *Pdu-Fezf* being in the ventral half and *Pdu-Irx* occupying the dorsal half of the episphere (Marlow et al., 2014); however, the interface of these genes is not conserved even in the ZLI homologue of hemichordates (Pani et al., 2012).

The apical organ might be related by its position and gene expression signature of *Pdu-FgfR* and *Pdu-Sfrp* (Marlow et al., 2014) to another organizer of the vertebrate brain

development, the anterior neural ridge (Pani et al., 2012). Neurons in the central part of the episphere that express *Pdu-Otx* (Figure 21, Figure 27), *Pdu-Six3* (Figure 21) and *Pdu-Nk2.1* (Figure 22, Figure 27) probably correspond to the diencephalon (cf. with the section 7.2.3.3). *Nk2.1* is a marker of the ventral hypothalamus and the ventral telencephalon (Pera and Kessel, 1997; Shimogori et al., 2010) and is required for hypothalamus development (Kimura et al., 1996). It was shown previously to co-specify conserved hypothalamic neurosecretory cell types in *Platynereis* (Tessmar-Raible et al., 2007). Lateral parts of the episphere positive for *Pdu-Wnt* genes [Figure 5 and (Pruitt et al., 2014)], *Pdu-Tcf* (Figure 11), *Pdu-Pax6* (Figure 20), *Pdu-Six3* (Figure 21) and lateral *Nk2.1* domains (Figure 22, Figure 27) would be then homologous to various parts of the vertebrate telencephalon (cf. with the section 7.2.3.3). This was already demonstrated for the mushroom bodies of *Platynereis* and the vertebrate pallium (Tomer et al., 2010). The regions of lateral *Pdu-Nk2.1* domains might be homologous to the vertebrate medial ganglionic eminences that in vertebrate brain development give rise to paired ventral basal ganglia known as globus pallidus which require *Nk2.1* for their development (Sussel et al., 1999). In the vertebrate brain, some *Nk2.1* positive cells migrate from the pallidum to the striatum where they produce cholinergic neurons (putative motor neurons) and to the pallium where they give rise to GABAergic interneurons (Sussel et al., 1999). A sudden appearance of the lateral *Pdu-Nk2.1* domains could thus be connected to the development of the trunk musculature and its motoric innervation in the metatrochophore and nectochaete stages. GABAergic neurons have been however so far observed only in the central part (also expressing *Pdu-Nk2.1* – Figure 27) of the *Platynereis* brain (Tomer et al., 2010).

A true evaluation of these proposed homologies will require further thorough experimental examination. Since a gene regulation and expression patterns also evolve, the congruence of organizers' fingerprints and hence their properties may not be absolute. These hypotheses imply that the peristomium of ciliated larvae is homologous to the collar of Hemichordata and in its ventral part also to the midbrain of vertebrates. The organizing centres in the CNS of vertebrates and insects according to them represent descendants of former ciliary belts (note that also the posterior edge of the peristomium bears ciliary belts – section 7.3.3.2). They have lost their cilia in some groups but kept their function as organizers that divide embryonic tissues to functional compartments with a specific gene expression. The signalling centres of the vertebrate and insect brains are hence a legacy from the times when their ancestors possessed ciliary belts.

11.2.2. Medio-lateral patterning

So far I attempted to assess homology between the *Platynereis* neuroectoderm and parts of the vertebrate brain but I put aside the question what is the role of Wnt/β-catenin in its CNS development. The data on A-P patterning offered too few clues to formulate a valid hypothesis. To answer it, we have to combine them with the data on the medio-lateral (M-L) patterning of *Platynereis* neuroectoderm with the knowledge of CNS development in other organisms.

11.2.2.1. A common theme of Platynereis A-P and M-L neural patterning

One common theme emerged from all *in situ* hybridization stainings of the expression of either A-P and M-L patterning genes that I have done after pharmacological treatments of Wnt/β-catenin pathway. It is the unsettling similarity of changes in gene expression between the Wnt activating and inhibiting conditions. The vast majority of inspected CNS patterning genes (in fact, all of those for which the more reliable and stronger inhibition by IWR-1-endo was done), namely *Pdu-Ngn2*, *Pdu-Pax6*, *Pdu-Six3* (in the episphere), *Pdu-Otx*, *Pdu-Nk2.1*, *Pdu-Emx*, *Pdu-Hox1* and *Pdu-Hox4* showed a decreased expression and/or reduced size of their expression domains upon either the ubiquitous pharmacological activation or inhibition of Wnt/β-catenin pathway. It is especially surprising for *Pdu-Ngn2* because *Neurogenin* is a direct target of Wnt/β-catenin signalling in mammals (Hirabayashi et al., 2004). One could thus expect that it will be elevated by an overactivated Wnt/β-catenin signalling. In fact, any gene controlled directly by Wnt/β-catenin signalling should display downregulation of its expression when the activity of the pathway is misregulated in one direction and upregulation in the other. More complex indirect regulation with an involvement of additional activators or inhibitors could potentially under certain circumstances yield similar result. But this would certainly not be the case of all the genes.

In neither case the expression domain was shifted, it always remained in a correct position, although a segmental organization in the trunk, if it was present in controls, was lost (e. g. *Pdu-Emx* – see the chapter 11.3 for discussion). In others (e. g. for *Pdu-Pax6*, *Pdu-Six3* in the episphere, *Pdu-Nk2.1*) the expression domains were reduced in size after pharmacological treatments of the Wnt/β-catenin pathway or even disappeared completely

(e. g. most of the *Pdu-Pax6* expression in the hyposphere upon CHIR99021 treatment, segmental expression in *Pdu-Otx*, episphere expression of *Pdu-Emx* in either treatment etc.). Again, this would make sense if the gene was regulated by Wnt/β-catenin signalling and the reduction in domain's size was observed under one condition whereas the other would result in its expansion. However, this was not the case and the decrease and reduction was observed under either condition.

The reduction could be explained as that the expression domain had retracted on its edges because the expression is lowest here and hence most affected by an overall decrease. Smaller and/or weaker expression domains would disappear completely. However, the premise of the weakest expression on the edges is disputable and more importantly, this hypothesis does not explain why a decrease is observed after either the activation or the inhibition of Wnt/β-catenin signalling. Is there any explanation for such strange behaviour of gene expression in response to manipulation of Wnt/β-catenin pathway?

11.2.2.2. A new model for the Wnt/β-catenin signalling function in the CNS development and evolution

The available data hence show convincingly that vertebrates, insects and annelids use orthologous transcription factors expressed in in identical order to pattern their neuroectoderm along the dorso-ventral (vertebrates) or lateral-to-medial (insects and annelids) axis. The system for D-V/M-L patterning of neuroectoderm is hence conserved across all three major clades of Bilateria and has been probably present already in their last common ancestor (Arendt et al., 2008).

But there is also a marked difference which was so far left unnoticed or neglected by previous authors. This conflict lies in the positions of signalling centres in the developing central nervous systems. The ventral body side in protostomes is according to the well-supported theory of D-V axis inversion in the chordate lineage homologous to the dorsal side of chordates (DeRobertis and Sasai, 1996; Nübler-Jung and Arendt, 1994). A second inversion of axis occurs in the nervous system of chordates during neurulation. The ventral neuroectodermal midline in protostomes like *Platynereis* thus corresponds to the ventral neural midline in chordates, i. e. the glial floorplate. The floorplate, as well as chorda produces Sonic hedgehog similar to the ventromedian axochordal muscle of *Platynereis* (Lauri et al., 2014) but not Wnt proteins. Instead, Wnt proteins are produced in the dorsal neural tube together with BMP and oppose ventral Shh (the section 7.2.3.2). Neurulation

does not take place in *Platynereis* and the dorsal neural tube of chordates thus topologically corresponds to the lateral neuroectoderm of *Platynereis*, which is characteristic by the activity of BMP, but not Wnt. Instead, the source of Wnt proteins in *Platynereis* is positioned in the ventral midline immediately next to the Hh-expressing axochord, where it together with the Hh signalling opposes the lateral BMP activity (Demilly et al., 2013; Denes et al., 2007; Lauri et al., 2014). The ventral midline of *Platynereis* corresponds to the vertebrate ventral neural tube. The source of Wnt/β-catenin signal is thus located in the opposite position relative to other signalling centres in *Platynereis* than it is in vertebrates.

What could cause the switch in the position of the Wnt signalling centre? There is a positive feedback between Wnt and Hh in protostomes as documented by the (para)segmental boundary (section 7.2.2.2). On the other hand, Wnt and Hh signalling became mutually antagonistic in vertebrates by regulating transcription of each other's inhibitors (Alvarez-Medina et al., 2008; Katoh and Katoh, 2006b). Yet the medial-to-lateral sequence of transcription factors expressed in the *Platynereis* neuroectoderm is the same as the ventral-to-dorsal sequence of chordates (Arendt et al., 2008), i. e. it is conserved despite the opposite position of the Wnt signalling centre. The evolution of Wnt-dependent patterning in both lineages would thus require multiple neural patterning genes to acquire an opposite type of regulation.

This discrepancy can be explained only if the Wnt/β-catenin signalling in at least one of these groups is not involved in the patterning of neuroectoderm at all and its primary mission is to promote neurogenesis. The patterning would be then dependent only on the conserved opposing BMP and Hh gradients. Is there any support that would justify such statements? And could this mechanism account for the observed gene expression phenotypes of Wnt/β-catenin pathway's activation or inhibition in *Platynereis* larvae?

Wnt/β-catenin signalling in vertebrates became associated by mutual activation with the BMP signalling in the dorsal neural tube, where they have opposing roles – Wnt/β-catenin signalling promotes cell proliferation and blocks differentiation and vice versa (Ille et al., 2007). Wnt/β-catenin signalling positively regulates the expression of proneural, cell cycle-progression, pro-proliferative and stemness genes in vertebrates (see the chapter 7.1.5) and promotes neurogenesis in the dorsal neural tube (Chesnutt et al., 2004; Megason and McMahon, 2002), the caudal midbrain (Panhuysen et al., 2004), the ventral telencephalon (Gulacsi and Anderson, 2008), the developing neocortex (Chodelkova et al., 2018) and in the adult hippocampus (Lie et al., 2005) during their development. Wnt

proteins are also able to stimulate either renewal and proliferation of neural stem cells both *in vitro* and *in vivo* (Kalani et al., 2008).

11.2.2.3. The neurogenic model of Wnt function explains the effects of Wnt pharmacological treatments

It was described previously in *Platynereis*, that the Pdu-Wnt4 protein produced by the ventral midline activates the Wnt/β-catenin signalling in the adjacent ectoderm and stimulates its neurogenesis (Demilly et al., 2013). The ventral midline in *Platynereis* embryos is formed by a fusion of blastoporal lips. They express *Pdu-Wnt4* already before the fusion at least from 20 hpf, possibly even from 16 hpf (Marlow et al., 2014) when the Wnt/β-catenin signalling might be involved in the specification of neural progenitors as evidenced in this stage by a transient expression of *Pdu-SoxB* (Kerner et al., 2009). The same process of commitment of neural progenitors probably takes place again later in segmental stripes of expression of *Pdu-Wnt4* and perhaps also in the expression domains of some other *Pdu-Wnt* genes, e. g. *Pdu-WntA* in the segments and the stomodaeum, during the metatrochophore stage (Demilly et al., 2013; Pruitt et al., 2014). All these tissues express either *Pdu-Wnt* genes as well as transiently *Pdu-SoxB* (Kerner et al., 2009). Committed neuronal progenitors cease to express *SoxB* and proliferate to generate naïve neurons.

The blastoporal lips are not yet fully fused in the early trochophore at 24 hpf and still express *Pdu-SoxB* (Kerner et al., 2009). The majority of neurogenesis (the proliferation of committed neuronal progenitors) in the central nervous system hence probably continues during and after the fusion of blastoporal lips. Consequently, the time window between 24 and 48 hpf which I used to activate or inhibit Wnt/β-catenin pathway in *Platynereis* larvae covered a substantial part of the neurogenic phase and could also interfere with the end of the specification phase of neural progenitors. As a result, less neuronal progenitors were specified and less naïve neurons produced when the Wnt/β-catenin signalling was inhibited in this neurogenic time period. Less undifferentiated neurons mean less material for neural differentiation and patterning with the expression the respective TFs. This explains the reduction of size of the expression domains upon Wnt inhibition. But why is the expression also weaker? And why we see the same situation with the Wnt activation?

We can assume that if high Wnt/β-catenin signalling maintains neural stem cells and stimulates their proliferation, the other side of the same coin is that it has to actually

prevent differentiation at the same time. To make the situation even more complicated, the roles of Wnt in the maintenance of stem cells and the promotion of proliferation are only seemingly in agreement and in fact are also contradictory because one of the characteristics of stem cells is that they are slow-cycling and generate fast-dividing transit amplifying progenitors on demand (Foudi et al., 2008; Furutachi et al., 2015; Johansson et al., 1999; Montgomery et al., 2011; Ono et al., 2012). Yet, both these features, proliferation and stemness, are promoted by canonical Wnt signalling. How is this possible? According to the traditional view, the stemness is maintained by signals from a stem cell niche which also slow down stem cell proliferation. I suggest an alternative view: a response to Wnt/β-catenin signalling might be dose-dependent and hence a high pathway's activity maintains stem cells but slows down proliferation, whereas intermediate Wnt levels promote proliferation but block differentiation and low or absent Wnt/β-catenin pathway's activity allows (or even contributes to) a differentiation. There is a simple mechanism of how such differential responses to the activity of a single signalling pathway can be achieved and I will include this explanation in the manuscript that is just being prepared. As a result, the artificial overactivation of Wnt/β-catenin signalling by CHIR99021 slowed down the proliferation of present neural progenitors and prevented their differentiation by keeping them in a more stem cell-like state. Therefore, there are less neurons after the activation of Wnt/β-catenin signalling and they express less or no differentiation markers of neural patterning.

Let's now get back to the last remaining question of why are the expression domains of patterning genes after the inhibition of Wnt/β-catenin pathway not only reduced in size but their expression is also weaker. Transcription factors expressed during patterning define distinct subpopulations of neural precursors and their expression becomes reduced in most differentiated neurons after they fulfilled their role. For instance, *Pdu-Pax6* is expressed in the entire ventral nerve cord and its commissures at 72 hpf (Figure 20) but only in few isolated cells at the bases of parapodia in the trunk of juvenile *Platynereis* atokous worm (Backfisch et al., 2013). Likewise, *Pax6* is expressed almost in the entire embryonic telencephalon of vertebrates but is confined just to several smaller neurogenic regions of the brain in the adulthood (Stoykova and Gruss, 1994). If an inhibition of Wnt/β-catenin signalling causes a premature differentiation it could then lead also to a premature loss of expression of some CNS patterning genes. Alternatively, an inhibition of Wnt/β-catenin pathway might cause a loss of pro-neural characteristics in neuronal precursors and these would be then unable to further differentiate and express subtype-specific genes.

The decreased number of commissures that connect the two ventral nerve cords (zero or one instead of usual three at 48 hpf stage) has been connected to an incomplete closure of the blastoporal lips which contain the VNCs for Wnt inhibition (Demilly et al., 2013). Here I demonstrate that the overactivation of Wnt/β-catenin pathway has the same effect, which indicates that it might interfere with the closure of the blastopore as well. This could happen via a blocking of the non-canonical Wnt/PCP pathway, which is active later in the ventral neuroectoderm where it drives its convergent extension (Demilly et al., 2013; Steinmetz et al., 2007). Alternatively, it might be attributed also to the lack of commissural neurons due to a deregulated neurogenesis.

11.2.2.4. Possible objections to the neurogenic model of Wnt function in the CNS

Someone could argue that the patterning function may have been lost in *Platynereis.* However, a loss of Wnt patterning function in the *Platynereis* lineage does not seem plausible as the Wnt expression is seen also in the primitive streak. i. e. the prospective ventral neural midline of vertebrates before neurulation (see the section 7.2.1.3). The vertebrate condition thus appears to be derived. It is possible that the Wnt function in the neural patterning, initially shared with Hh signalling, has been lost in contrast in the vertebrate lineage or that it never existed. In these two cases, all effects of Wnt manipulation on the neural patterning in vertebrates should be reinterpreted with respect to shifts of neural cells to more (in the case of Wnt inhibition) or less (in the case of Wnt activation) differentiated state and/or to Wnt interactions with the Shh and BMP signalling pathways. This is quite unlikely since there are numerous studies that document the function of Wnt in neural patterning in vertebrates. Of course, there is the last possibility that the existing Wnt gradient has been independently co-opted for neural patterning in vertebrates.

Possible conflicting findings in vertebrates and *Platynereis* about the effect of the Wnt/β-catenin pathway on patterning could be hence explained by a co-option of Wnt/β-catenin signalling for CNS patterning in the vertebrate lineage. Because Wnt/β-catenin was usually activated or inhibited conditionally in the experiments with vertebrates and enough neuronal progenitors were already present, a putative patterning function could be revealed. The timing of this conditional activation or inhibition may shift the balance between the neurons in a different stage of differentiation and hence also the corresponding expression patterns, similar to the conflicting result in *Platynereis* itself which are discussed in the next

paragraph. This could happen especially if the Wnt/β-catenin pathway was activated or inhibited topically which is common in vertebrates, whereas I used the whole body manipulation of the Wnt/β-catenin pathway's activity in the whole body.

There is an apparent conflict between my data on *Pdu-Ngn2* expression after IWR-1-endo inhibition between 24 and 48 hpf (Figure 19) and those obtained by Demilly et al. after the inhibition from 33 hpf to 55 hpf (Demilly et al., 2013). Whereas I observed a significant decrease in the *Pdu-Ngn2*-expression, Demilly and colleagues report an increased number of *Pdu-Ngn2*- and EdU-positive cells, but a decreased total number cells. Similarly, the activation of Wnt/β-catenin pathway by CHIR99021 from 12 hpf to 24 hpf caused an expansion of the *Pdu-Pax6* expression in the episphere of 24 hpf early trochophores (Marlow et al., 2014), whereas I did not observe any marked changes in the episphere expression after the treatments form 24 to 48 hpf and the expression was decreased in the hyposphere (Figure 20). The blame may be on the time period and hence the developmental stage used for pharmacological treatments. Whereas elevated Wnt/β-catenin signalling before 24 hpf stage probably led to an increased specification of neural progenitors, the inhibition of the same pathway later in development might cause neural stem progenitors to lose their stemness and divide symmetrically to naïve neurons.

I can exclude that the earlier effect of Wnt pathway manipulation on neurogenesis could conceal a later effect of Wnt/β-catenin signalling on patterning in *Platynereis*. This might not be uncovered in my experimental set-up in the case that both events would take place between 24 and 48 hpf in *Platynereis* when the pharmacological treatments were done. However, the residual expression after the treatments did not show any signs of shifts of the expression domains. Therefore, my data support the idea that the sole function of Wnt/β-catenin signalling in the neuroectoderm of *Platynereis* is to generate neural progenitors and general undifferentiated neurons, but it is not involved in neural patterning. It is plausible that this condition in *Platynereis* is primitive, whereas the vertebrate situation is derived and this mechanism thus could represent an evolutionarily ancestral role of Wnt/β signalling in neural development.

11.3. Segmentation

11.3.1. Wnt/β-catenin signalling in the *Platynereis* larval (primary) segmentation

11.3.1.1. The segmentation mechanism is conserved between insects and annelids

It has been proposed before that the segmentation mechanism on the segmental boundary of *Platynereis* larval (primary) segments is identical to the system of the establishment of parasegmental boundaries by segment polarity genes in *Drosophila* (see the section 7.2.2.2). These predictions were based on the segmental expression patterns of *Pdu-Engrailed, Pdu-Wnt* genes, members of the Hh pathway and effects of the pharmacological inhibition of Hh pathway by cyclopamine (Dray et al., 2010; Prud'homme et al., 2003). The evidence however lacked a proof that Wnt/β-catenin signalling positively regulate the expression of *Pdu-En* and *Pdu-Hh*.

The pharmacological activation of Wnt/β-catenin signalling (Figure 24) clearly confirmed that *Pdu-En* expression is positively regulated by the canonical Wnt pathway. Yet, despite the expression of *Pdu-En* was stronger after the activation, it remained confined to their segmental stripes. Therefore, there has to be a very efficient system in place that limits the Wnt activity and *Pdu-En* expression. Such situation exactly corresponds to the border between parasegments in *Drosophila*, where Wingless induces expression of *En* and *Hh* in the adjacent row of cells but the Wnt-dependent phosphorylation of Cubitus interruptus cause its proteolysis and to prevents the activation of Hh signalling in the very same cells (Jia et al., 2002; Price and Kalderon, 2002). Moreover, Wingless signalling also blocks the transcription of *Ci* gene. An analogical situation occurs in Wg-producing cells in response to Hh signal (see the section 7.2.2.2). Both pathways thus activate each other in a positive feedback loop but they are also mutually exclusive at the same time. A weak mutual activation in an autocrine manner is unstable fluctuations in the gene expression are reinforced to the state when the positive feedback between Wg and Hh is purely paracrine. The result is a formation of a sharp boundary between the anterior Wg-producing/Hh-responsive/Wg-non-responsive and the posterior Hh-producing/Hh-non-responsive/Wg-responsive cells. Therefore, once the segmental pattern was established, the stronger is one of the signals, the stronger is the response from the other. The stronger Wnt signalling in *Platynereis* therefore probably

could act only on the Wnt-responsive *En*-expressing, Hh-producing cells, which caused proportionally stronger inhibition of Wnt signalling in the Wnt-producing cells and prevented the activation of *Pdu-En* expression in their stripes.

Although the data from *Pdu-Hh* expression in response to a manipulation of Wnt/β-catenin signalling are still lacking, we can reasonably assume that the regulatory relationship between Wnt and Hedgehog pathways on the segmental/parasegmental boundary is conserved between *Platynereis* and *Drosophila* and a mutual activation occurs between Wnt/β-catenin and Hh signalling on the segmental boundary of the primary segments in *Platynereis*.

11.3.1.2. A theoretical model for the primary segmentation in Platynereis larvae

It might be intuitively presumed that the segmental expression pattern of many genes could be hardwired in the stereotypic development of *Platynereis* and the cells in segments are committed to express certain genes. If such cell would be removed and transplanted elsewhere in the embryo, it should then express the same set of genes regardless of its new embryonic context, as would be expected from a deterministic development. As to my knowledge, such transplantation has never been done in *Platynereis*.

At this place, I would like to note that although the terms deterministic and stereotypic development are often used interchangeably, they actually have different meanings. The term "stereotypic development" refers to the fact that same cells are always located in the same position within the body and relative to each other, while the term "deterministic development" refers to the early and absolute commitment of cell fate, i. e. the gene expression, not the position. Although the development of *Platynereis* is with no doubts stereotypic, I do not know about any (recent) transplantation experiments with larvae. Transplantation of parapodia in adults showed tissue commitment, nevertheless this was a long time after the embryonic development and commitment of adult tissues is quite common even in organisms with regulative development. The early development of *Platynereis* is deterministic in a way that cells have unique patterns of Wnt/β-catenin signalling activation (and hence gene expression) in their lineages and the cell fates are narrowed down with every other cell division. However, this lasts only until the onset of bilateral symmetry. Although the cell lineages later still have fixed fates and even gene

expression, it is not clear whether this reflects the deterministic development by a segregation of cytoplasmic factors during unequal cell divisions or if it is just a consequence of the stereotypic development, which causes always the same cells in the lineage to assume exactly the same positions in the body and respond to the same signals. The early deterministic development could just serve to create signalling centres and responsive/permissive cell populations for later regulative, but constrained and stereotypic development. This view is supported by the fact that the entire ectodermal lips gastrulating by epiboly in *Platynereis* are descendants of a single symmetrically cleaving blastomere $2d^{112}$ after a termination of unequal cell divisions which is regulated by asymmetrical activation of Wnt/β-catenin pathway (Schneider and Bowerman, 2007). This could apply not only for *Platynereis* but for all organisms – their developments can differ only in lengths of deterministic, regulative and stereotypic phases. For these reasons, I consider the type of development in *Platynereis* at the critical time of segmentation as uncertain and propose an alternative explanation of primary segment formation.

Pdu-Wnt genes and also *Pdu-Tcf* (section 10.1.5.2) are initially expressed ubiquitously and homogenously, but weakly throughout the hyposphere of the early trochophore larva at 24 hpf (Pruitt et al., 2014). Based on our knowledge of the regulatory relationship between Wnt and Hh signalling later in the development on the segmental boundary, the outcome of such weak ubiquitous activity of Wnt would be naturally also weak ubiquitous production and activation of Hh. If we make one more assumption supported by the data from *Drosophila* (see the section 7.2.2.2) that at least one of this pathways at the same time represses expression of its own ligands in the target cells, we will get an unstable equilibrium of Wnt and Hedgehog that will spontaneously form a pattern according to the Turing's reaction-diffusion model (Turing, 1952). In this case, the interactions between Wnt and Hh signalling would reinforce fluctuations in expression until they would establish stationary waves – the segmental stripes. Their width would be determined by diffusion rates of Wnt and Hh and by their reaction coefficients, i. e. their abilities to regulate gene expression. The area of ventrolateral neuroectoderm of *Platynereis* larva of given stage is definite, limited and due to the stereotypic development also of constant size with a constant Wnt expression in the posterior growth zone, ventral blastoporal lips and anterior domains in the lateral episphere that create the nodes to anchor the stationary expression waves and determine their direction. Hence the number and position of the stripes would be always the same, creating an illusion of deterministic development.

11.3.1.3. Wnt/β-catenin signalling in the development of chaetae

The overactivation of Wnt/β-catenin pathway leads to an abrogation of chaetae development, which was shown to depend on Delta-Notch signalling (Gazave et al., 2017). I suggest that the ability of Wnt/β-catenin signalling to activate the expression of Delta ligands and activate Notch signalling (7.1.5.2) is essential for the development of chaetae.

11.3.1.4. Wnt/β-catenin signalling does not determine segment identity

The absence of anterior or posterior shifts of *Pdu-Hox* gene expression after any of the manipulations of Wnt/β-catenin pathway suggests that the Wnt signalling is not involved in the A-P determination of segment identity (section 7.2.2.4). In vertebrates, *Hox* genes are controlled by retinoic acid, ParaHox proteins and may be regulated by Wnt/β-catenin directly (section 7.2.3.3) or via the transcription control over *ParaHox* genes by Wnt (7.1.5) or FGF signalling (Isaacs et al., 1998). Only the regulation by Wnt/β-catenin signalling is present in *C.elegans*, whereas insects use the activation of *Hox* genes by maternal genes including *Cdx*/*caudal* and pair-rule genes (section 7.2.2.4).

Pdu-Hox genes are not regulated by retinoic acid (Handberg-Thorsager et al., 2018) or Wnt/β-catenin signalling. On the other hand, *Pdu-Cdx* is a positive target of the Wnt/β-catenin pathway (section 10.4.3.1). It may thus seem that only remaining option for *Hox* regulation in *Platynereis* is a regulation by FGF via *ParaHox*. But why would not the *Pdu-Hox* genes react to the regulation of *Pdu-Cdx* by Wnt/β-catenin signalling? If we consider this, we have to eliminate all options for *Hox* regulation known from vertebrates. A solution for this conundrum might lie in the stage when the effect of signalling pathways was tested – the canonical Wnt signalling may be important only to establish the expression of *Pdu-Hox* genes early in development but not for its maintenance, similarly to its requirement for the establishment of early *Hox* expression in vertebrates (Bouillet et al., 1996; Nordström et al., 2006). The regulation of *Pdu-Hox* gene expression could be possibly mediated by Wnt-dependent regulation of *Pdu-Cdx* and other *Pdu-ParaHox* genes earlier during development before the pharmacological treatments were done. The regulation of *Pdu-Hox* genes by Wnt/β-catenin signalling directly or via *ParaHox* genes is supported by the expression of multiple *Pdu-Wnt* genes (Janssen et al., 2010), *Pdu-Hox* genes (Novikova et al., 2013; Pfeifer et al., 2012) and *Pdu-Cdx* (de Rosa et al., 2005; Kulakova et al., 2008) during posterior regeneration in nereidids.

11.3.2. Wnt/β-catenin in *Platynereis* post-larval segmentation

11.3.2.1. Wnt/β-catenin signalling functions in the posterior segment addition zone

Wnt signalling has been proposed to involved in the sequential addition of new segments in the segment addition zone (SAZ) during normal development and regeneration of *Platynereis*. It is often involved in this process in other animals (see the section 7.2.2.1) and the *Platynereis* SAZ is in a close proximity of the Wnt signalling centre observed in the pygidium (Janssen et al., 2010). Expression of *Pdu-Axin* and *Pdu-Tcf* in the SAZ shown here (Figure 7, Figure 13, Figure 14) provides the indirect evidence that the Wnt/β-catenin pathway is active in the SAZ of *Platynereis*.

The expression of Wnt components in the posterior SAZ overlaps with that of *Pdu-Cdx* [Figure 28 and (de Rosa et al., 2005; Kulakova et al., 2008)]. The strength of *Pdu-Cdx* expression is dependent on the activity of the Wnt/β-catenin pathway (Figure 28) and the same is true for *Pdu-Tcf* expression in the SAZ (Figure 14), which suggests that *Pdu-Cdx* and *Pdu-Tcf* are positively regulated by the canonical Wnt signalling. At the same time either the posterior *Pdu-Wnt1*, *Pdu-Wnt11* and *Pdu-Cdx* are not disrupted by the inhibition of Hh signalling unlike the more anterior segmental *Pdu-Wnt* expression (Dray et al., 2010). Together these data indicate that a positive feedback loop between Wnt/β-catenin signalling and Pdu-Cdx and/or another protein, e. g. Pdu-Brachyury (Martin and Kimelman, 2008), may exist in *Platynereis,* which maintains Hh-independent posterior *Pdu-Wnt* expression. Reactivation of Wnt signalling in the regeneration blastema may thus play a central role in the establishment of *Pdu-Cdx* expression and of a new posterior growth zone, formation of a new pygidium and a reconstitution of SAZ after tail amputation. In agreement with this statement, a reactivation of the canonical Wnt signalling is crucial for the formation of a regeneration blastema in planarians (Gurley et al., 2010; Petersen and Reddien, 2009b), the caudal fin of fish (Poss et al., 2000; Wehner et al., 2014) and during either the tail (Lin and Slack, 2008) and the limb regeneration in amphibians (Yokoyama et al., 2007).

Generally speaking, new segments are specified by oscillations in gene expression induced by segmentation clock which involves Wnt/β-catenin signalling (section 7.2.2.1). If the *Pdu-Wnt* expression in the regenerative blastema or pygidium reaches approximately to the SAZ, these oscillations could in fact represent periodic anterior-posterior back and forth fluctuations of the *Pdu-Wnt* anterior boundary due to feedback regulatory interactions of Wnt/β-catenin pathway with other genes or signalling pathways.

11.4. Wnt/β-catenin signalling in the *Platynereis* gut development

11.4.1. Wnt/β-catenin signalling is necessary for the gut differentiation in *Platynereis dumerilii*

11.4.1.1. Inhibition of Wnt/β-catenin signalling functionally converts the midgut to the hindgut in Platynereis

The alimentary canal of *Platynereis* consists of three functional and morphological compartments – from the anterior foregut formed by the muscular pharynx, the central midgut and the posterior hindgut. The expression of the extracellular digestive enzymes reflects functional difference between gut compartments. The midgut compartment is undoubtedly dedicated for the extracellular digestion as evidenced by its expression of the genes for extracellular peptidases *Pdu-Subtilisin-1* and *Pdu-Subtilisin-2* and an extracellular polysaccharide digestive enzyme *Pdu-α-Amylase*, whereas the expression of these extracellular digestive enzymes is absent in the hindgut On the other hand, a presence of the intracellular digestive enzyme *Pdu-Legumain* might reflect that this compartment is specialized for the absorption and processing of products from the midgut digestion. (Figure 26, summarized in the Figure 33).

Interestingly, the activation of Wnt/β-catenin signalling did not cause any change of expression of these digestive enzymes. This fact alone indicates that the activation of Wnt/β-catenin pathway does not have an instructive, but only permissive role in the differentiation of the gut compartments. Conversely, the inhibition of the canonical Wnt signalling led to a loss of expression of the midgut-specific enzymes and to the expansion of *Pdu-Legumain* from the hindgut to the midgut and the larval nephridia (Figure 26). The digestive epithelium of the *Platynereis* midgut is thus functionally converted to the hindgut-like epithelium (Figure 34).

This is in a sharp contrast with the situation in most other animals – posterior Wnt signal mediated by Cdx is necessary to specify posterior and to suppress anterior fate in vertebrate gut [(McLin et al., 2007; Sherwood et al., 2011), compare (Beck et al., 1999) with (Lickert et al., 2000)] and it is essential for the development of hindgut in *Drosophila* (Lengyel and Iwaki, 2002; Wu and Lengyel, 1998) or the beetle *Tribolium* (Oberhofer et al., 2014) (see also the section 7.2.4.2). Indeed, the activation of Wnt/β- catenin signalling

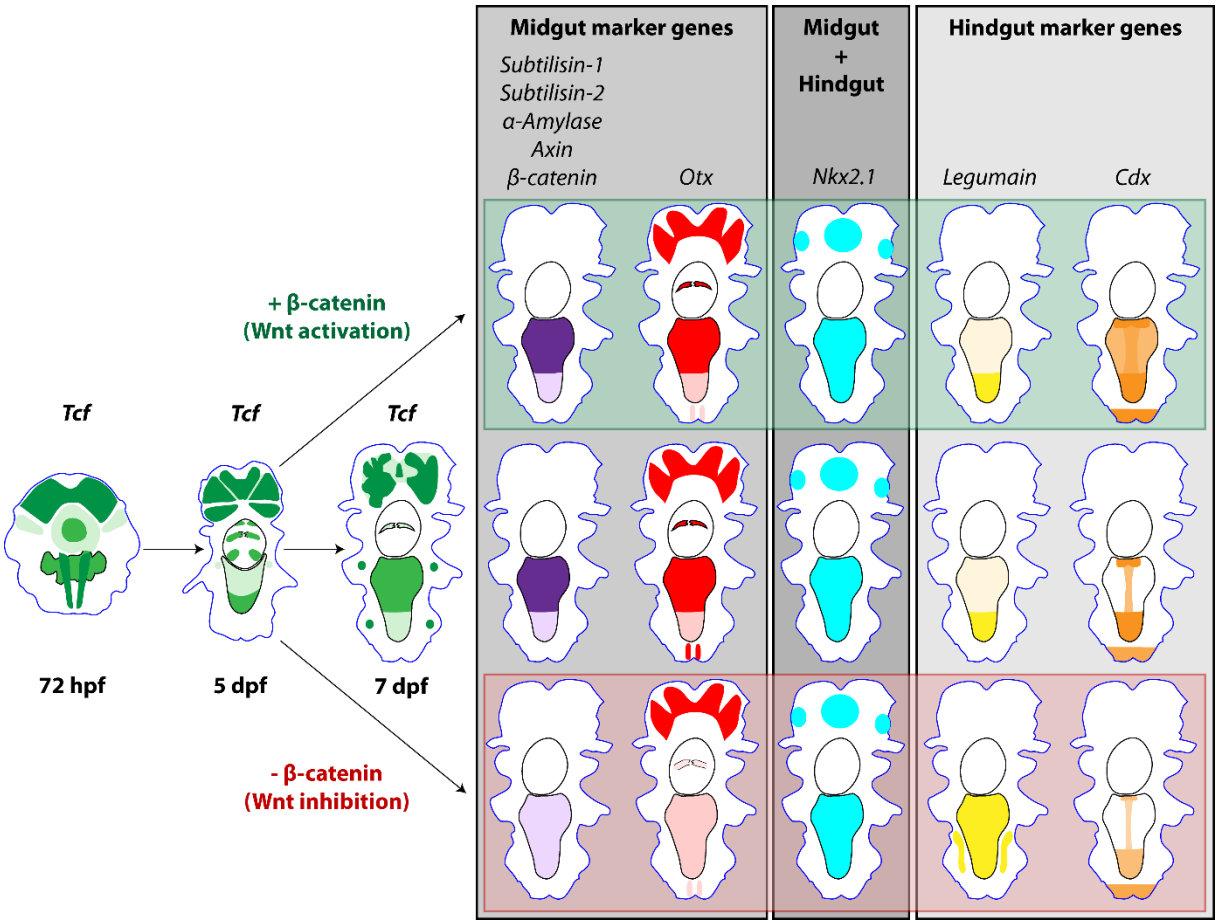

Figure 33 – Recapitulation of Pdu-Tcf and gut-marker genes' patterns upon Wnt/β-catenin manipulation

The scheme summarizes the expression of *Pdu-Tcf* of the 72 hpf, 5 dpf and 7 dpf stages and the normal expression of gut specific marker digestive enzymes and transcription factors and their changes upon the pharmacological activation or inhibition of Wnt/β-catenin pathway.

Regarding the gut, *Pdu-Tcf* is expressed in the stomodaeum at 72 hpf stage, in the hindgut and isolated cells of the foregut but only weakly in the uncellularized midgut of the 5 days old larva, and in the midgut, which is now part of a fully compartmentalized functional digestive tract at 7 dpf.

Midgut marker genes are in general downregulated by the inhibition of Wnt/β-catenin signalling whereas hindgut marker gene *Pdu-Legumain* expands to midgut, while no major changes are observed upon Wnt/β-catenin activation. Therefore, the expression profile of these genes in the midgut changes upon Wnt inhibition to the one that is reminiscent of the hindgut, and Wnt/β-catenin seems to have only permissive, not directive role in gut differentiation.

On the other hand, another hindgut gene *Pdu-Cdx* is slightly downregulated upon inhibition and expands after activation, suggesting different mode of regulation of this early patterning gene with an instructive/directive role of Wnt/β-catenin signalling *Pdu-Cdx* activation.

The expression of a more general endodermal marker *Nk2.1* is not changed by any of the manipulations of Wnt/β-catenin pathway, consistent with its normal presence in both gut compartments.

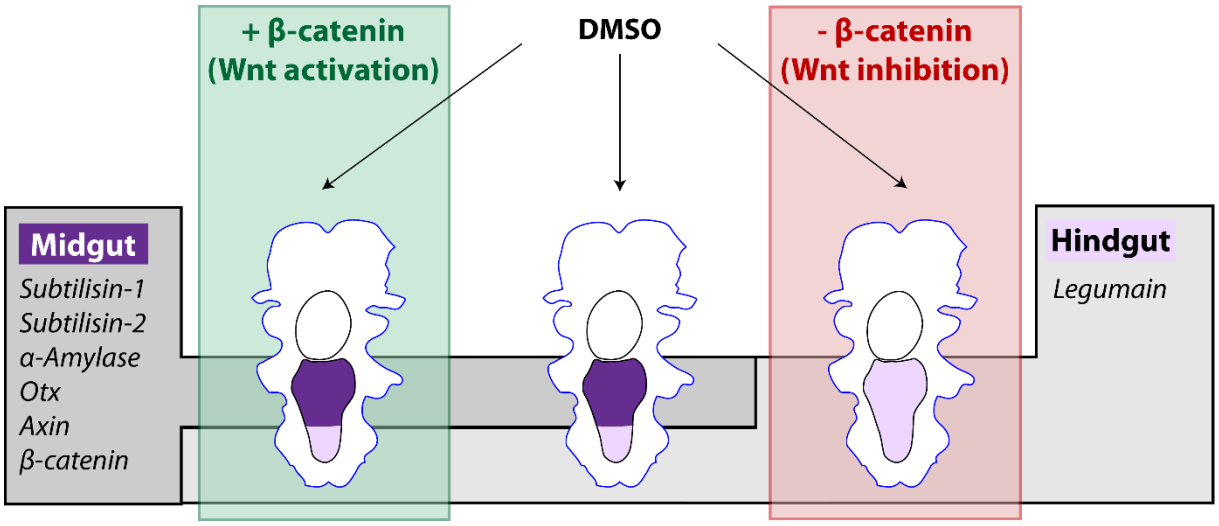

Figure 34 – Model of gut differentiation regulation by Wnt/β-catenin signalling

Here, I propose a model in which Wnt/β-catenin signalling pathway is active in the midgut (as supported by the presence of *Pdu-Axin* and Pdu-β-catenin and the activity of Wnt-responsive transgenic construct), where it has an indispensable permissive role it gut precursor cells proliferation and in the differentiation of the precursors into a functional midgut secretory epithelium. The inhibition of Wnt/β-catenin pathway causes midgut cells to lose their midgut fate and convert by their expression profile (and hence probably also functionally) to the hindgut-like tissue. At the same time, Wnt/β-catenin signalling is actively inhibited or lacks other necessary factors to promote midgut fate in the hindgut since Wnt/β-catenin activation does not cause the hindgut tissue to differentiate into midgut.

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is able to shift the anterior boundary of the *Pdu-Cdx* expression in the hindgut towards the anterior into the midgut (Figure 28). However, as I already pointed out, it is not accompanied by any similar expansion of the hindgut differentiation marker *Pdu-Legumain* or retraction of posterior border of midgut markers. Instead, an anterior expansion of *Pdu-Legumain* expression and the adoption of posterior fate by the midgut is observed upon an inhibition of Wnt/β-catenin pathway (Figure 26, Figure 33, Figure 34). This is a real riddle, because the expression of the differentiation markers reacts in the opposite way than that of the patterning gene in response to a manipulation of the signalling pathway.

What could be then the role of Wnt/β-catenin signalling in the *Platynereis* gut development that would explain all of the observed facts? If the acquired data do not fit into the model of Wnt-dependent posterior patterning then (although it might be strange on the first look) I suggest that the Wnt/β-catenin signalling is not involved in the A-P patterning of the *Platynereis* gut at all.

11.4.1.2. Does Wnt/β-catenin signalling regulate gut A-P patterning and compartmentalization?

Before we can answer this question, it is necessary to clearly define the terms and to be aware of their difference. an A-P patterning can be defined as a subdivision of a tissue into molecularly distinct territories along the anterior-posterior axis by the expression of specific sets of transcription factors and their target genes, usually in response to the activity of a signalling pathway(s). On the other hand, a compartmentalization is a composition of the organ from functionally and morphologically distinct subunits. A compartmentalization often results from a previously specified molecular pattern as in the case of functional compartments in the vertebrate midgut, which differentiates into the stomach, the duodenum, the liver with the gall bladder, the pancreas and the small intestine However, in some cases it can be also the other way around and a pattern can result from a compartmentalization.

The gut compartments of *Platynereis* display distinct gene expression, but they arise from the fusion of the three independent primordia – the foregut invagination, the midgut primordium and the hindgut invagination – and not in response to the A-P molecular patterning. Unlike in *Drosophila*, in which the tripartite gut arises in a very similar way to *Platynereis* but later differentiates into more functional compartments (Buchon et al., 2013), the midgut of *Platynereis* does not show any signs of further molecular or morphological subdivision along its A-P axis in larvae. Regular constrictions of the gut in each segment in the atokous worms may result from spatial constraints because of other segmental organs and segmental septa and do not necessarily represent true compartments. This is supported also by a uniformous expression of *Pdu-Tcf* in the gut (Figure 16). The A-P compartmentalization of the gut in *Platynereis* is thus a consequence of its embryonic formation and not of molecular patterning. Therefore, whatever the role Wnt/β-catenin signalling plays in the early gut development of *Platynereis*, it is not (Prud'homme et al., 2003)the A-P patterning.

All three gut compartments in *Platynereis* express *Pdu-Tcf* and *Pdu-Wnt* genes at some point in their development. First, *Pdu-Tcf* becomes apparent in the future stomodaeal region at 24 hpf (Figure 14) and either canonical and non-canonical *Pdu-Wnt* genes begin to be expressed in the stomodaeum [Figure 5, (Dray et al., 2010; Pruitt et al., 2014)] as well as *Pdu-Axin* (Figure 6) and *Pdu-Tcf* (Figure 11, Figure 12) are strongly transcribed in the stomodaeum before 48 hpf and according to *Pdu-Tcf* they persist there until at least 72 hpf,

in isolated cells up to 5 dpf. The prospective hindgut starts to express *Pdu-Wnt* genes already before invagination in the pygidium from 24 hpf or even earlier (Pruitt et al., 2014). Also *Pdu-Axin* and *Pdu-Tcf* are present in the posterior of 48 hpf larva and *Pdu-Tcf* is detected in the hindgut invaginations also in the 72 hpf and 5 dpf larvae. However, all these genes for components of the Wnt/β-catenin signalling pathway cease to be expressed in the foregut and hindgut. Instead, either *Pdu-Axin* (Figure 7) and *Pdu-Tcf* (Figure 11-Figure 14) become strongly expressed in the midgut on the day 7 of development and the high levels of β-catenin protein, that are present here, suggest that the Wnt/β-catenin is active in the midgut. In summary, Wnt/β-catenin signalling is present during the formation of all three parts of the gut but is for some reason becomes activated later in the midgut primordium. I suggest that non-canonical Wnt signalling triggers and orchestrates the invagination of the stomodaeum and the hindgut. But what is the role of the canonical Wnt/β-catenin signalling pathway in gut development?

11.4.1.3. A theoretical model for the function of Wnt/β-catenin signalling in the specification and proliferation of endodermal precursors

Wnt/β-catenin signalling is on the top of the programme for the commitment of the endodermal progenitors both in development (section 7.2.4.1) and the adult homeostasis (section 7.2.4.3) in many organisms and hence has to be active in all naïve endodermal precursors. At the same time, an activation of the Wnt/β-catenin pathway in vertebrates upregulates the expression of genes for a progression through the cell cycle and for stemness programme (chapter 7.1.5). Wnt/β-catenin signalling thus functions in other animals in the specification and proliferation of the endodermal progenitors.

The inhibition of Wnt/β-catenin signalling completely abrogates cell proliferation in *Platynereis* larvae (Figure 29, Figure 30) and leads to a loss of differentiated digestive epithelium. This suggest that it may confer the same function in also in *Platynereis*. However, I did not see expression of Wnt pathway's components in the midgut primordium until 5 dpf (I interpret the *Pdu-Tcf* positive region at 72 hpf on the as the hindgut invagination and possibly also the muscle mesoderm).

Wnt/β-catenin signalling can specify the foregut and the hindgut epithelia early in development, but the midgut endoderm is derived from macromeres – huge, originally vegetal cells completely filled with yolk and a single large lipid droplet. The macromere

lineage is specified by Wnt/β-catenin signalling early in the *Platynereis* development during the embryonic cleavage [(Schneider and Bowerman, 2007), see the section 7.3.5.1]. Then they stop dividing and remain dormant at least until the end of the metatrochophore stage at 75 hpf (Fischer et al., 2010). It is possible that macromeres use the lipids from their yolk to produce lipoprotein particles during this time and provide nutrition to the rest of the cells of the developing larva. This has however never been studied. Such lipoprotein particles, similar to LDL of vertebrates or lipophorins of insects, could help to facilitate a long-range spreading of Wnt signal in the surrounding ectoderm during the establishment of segmental pattern of expression by Wnt signalling (section 11.3.1.2) and neurogenesis (11.2.2.3). The macromeres eventually start to divide ("cellularize") in the nectochaete stage after 75 hpf and generate a midgut primordium which connects to the stomodaeal (pharyngeal) and proctodaeal (anal) invaginations to form a through gut (see also the section 7.3.7.3). It thus seems that the reactivation of Wnt/β-catenin signalling in the midgut primordium might be connected to the reactivation of the cell division.

As well known among developmental biologists, the yolk inhibits embryonic cleavage. The yolk might present a mechanical obstacle as generally perceived; however, I propose that the yolk-induced inhibition of embryonic cleavage is regulated by a signalling that is dependent on the cytoplasm-to-nucleus ratio. The most intuitive would be if the signal was triggered by the yolk itself, but I suggest that the cause might be again the Wnt/β-catenin signalling. It is possible that after first embryonic cleavages, the level of maternal β-catenin drops so low, that it is no longer sufficient to promote cell proliferation. The large cytoplasm volume and cell diameter of the macromeres does not allow sufficient accumulation of β-catenin. Once the cytoplasmic volume drops below a critical level, the cells are able to accumulate enough β-catenin and in response start to divide – at first slowly, but then progressively faster with decreasing intervals. Consequently, they also express Wnt target genes that commit them to the fate of undifferentiated endodermal gut progenitors – quite in a parallel to the function of Wnt in the generation of neural progenitors in the ectoderm (the section 11.3.1.2).

Interestingly, the amount of yolk produced by the annelid *Capitella* sp. is dependent on a diet and sibling larvae from a single batch can be either lecitotrophic or planktotrophic (Qian and Chia, 1992). To my knowledge, it is not known whether a similar dependence of the yolk production on a diet exists also in *Platynereis*. But it is highly plausible and it is probably the cause of variability in the expression of the midgut-specific genes that I observed at 7 dpf (Figure 26B). If the amount of yolk varies among individuals, the re-

activation of midgut development happens at a slightly different time and the treatment and the detection of gene expression are done in a different stage of the gut development.

How can this theoretical model cope with the observed changes in the expression of differentiation markers of a mature digestive epithelium?

11.4.1.4. The differentiation of the digestive epithelium in Platynereis

What is the next logical step after the activation of the Wnt/β-catenin pathway in the midgut primordium? We know from the study of segmentation in *Platynereis* that Wnt/β-catenin signalling should most probably activate Hh signalling – first ubiquitously in an ustable equilibrium of coactivation of both pathways. I did not observe a significant increase in the proliferation or tumours in the gut of *Platynereis* larvae after the pharmacological activation of the Wnt/β-catenin pathway (Figure 29). This suggests that an efficient system is in place which inhibits the overproliferation. This could be achieved by the mutually exclusive positive feedback with Hh signalling, whose inhibitory response is just as strong as the Wnt activation, as I already described for the *Platynereis* segments (section 11.3.1.1). At the same time, we know from vertebrates that Indian hedgehog signalling in mature cells between the crypts directs the differentiation of colonocytes from Wnt-positive endodermal intestinal precursor cells. If we put these pieces of informations together, we can speculate that the role of Hh signalling in the differentiation of the digestive epithelium might be conserved in the evolution and controls the maturation of the digestive epithelium in *Platynereis*. However, a co-activation of both these pathways in the same cells would represent an unstable state (cf. the sections 11.3.1.1 and 11.3.1.2). Moreover, if these pathways have opposite effects on the cell state, i. e. the Wnt/β-catenin signalling pushes cells toward undifferentiated/stem state whereas Hh signalling towards a differentiation, the net result would be a semi-differentiated state. Instead, a co-activation of Hh by Wnt/β-catenin signalling should lead to a formation of a boundary of a mutually exclusive activity and expression. However, the gene expression patterns observed in the gut at 7 dpf are homogenous. Why would not be there a pattern established in the if ubitqitous Wnt signalling should activate Hh in an analogical situation to the segments? There are several possible explanations for this discrepancy.

First, it is possible that the boundary between Wnt and Hh signalling is located between the mesoderm and endoderm of the gut wall, i. e. that Wnt proteins produced by mesodermal cells activate the Wnt/β-catenin signalling in the gut endoderm, this in turn

produce Hh protein and by activating Hh in turn stimulates the production of Wnt in the gut mesoderm. Digestive gut epithelium differentiates in response to Wnt signals emanating from the surrounding visceral mesoderm for example in the gut of *Drosophila* (Nakagoshi, 2005). This however does not make sense if the Wnt is to regulate the proliferation and specification of endodermal progenitors and of Hh to cause differentiation of the same cells.

A second option is that the regulatory relationship between Wnt and Hh is different in the endodermal context and a stable equilibrium between Wnt and Hh is achieved or Hh signalling replaces Wnt signalling later in development in differentiated digestive epithelium. The latter possibility is not favoured by the observation that *Pdu-Tcf* is strongly expressed in the digestive gut epithelium even in the feeding worms (Figure 16). However, the activity of Wnt signalling is dependent on the presence of a Wnt signal and Pdu-Tcf might function as a repressor of target genes in its absence (cf the section 7.1.4.4).

There is a chance that I observed the midgut expression of *Pdu-Tcf*, *Pdu-Axin*, digestive enzymes, *Pdu-Nk2.1*, *Pdu-Otx* and the β-catenin protein in the period of unstable equilibrium between Wnt and Hh signalling before any pattern could be established. It is true that I saw in some individuals three longitudinal rows (two lateral and one in the middle) of about 5 large cells each that expressed the digestive enzymes stronger than the rest of the gut. Some of these cells are apparent on the Figure 26 with the staining of *Pdu-Subtilisin-1* and *Pdu-Subtilisin-2* after the mild inhibition by JW55, when the expression in most of the gut had been lost but these stronger expressing cells retained some expression of these digestive enzymes. However, these cells represent rather a secretory subtype of digestive cells in the overall differentiated absorptive epithelium. However, Hh signalling rather directs Wnt-positive endodermal precursors to a generally differentiate to a digestive epithelium. Cell fate decisions among its subtypes would be more probably driven by a universally used mechanism of Delta-Notch signalling in an analogous way to the case of enteroendocrine vs. enterocyte cell fate decision in vertebrates (Zecchin et al., 2007) or the differentiation of secretory copper cells in *Drosophila* (Tanaka et al., 2007). In these organisms, secretory cells express Wnt and Delta/Jagged, which triggers the Notch signalling in surrounding cells and pushes them toward the absorptive fate (see the section 7.2.4.3 for citations).

Nevertheless, a closer examination of the *Pdu-Tcf* expression in the gut of the juvenile worm (Figure 16H) reveals, that there might be crypt-like formations in the gut epithelium of *Platynereis*. Unlike in vertebrates, the strongest *Pdu-Tcf* expression is found on the luminal side and a weaker on closer to the base with a gap with a few or almost no

Pdu-Tcf signal in the intermediate level. The most β-catenin protein is seen closer to the base of the epithelium together with a weaker *Pdu-Tcf* expression. These observations could be explained if the proliferation occurs at the base as in vertebrates (7.2.4.3) and Hh signalling on the luminal side drives the expression of a repressor isoform of *Pdu-Tcf* or the strong luminal *Pdu-Tcf* singal is actually probe trapping (the negative control for this experiment was not done).

It is possible that the Hh role in intestinal differentiation of vertebrates has been acquired secondarily as a result of a negative mutual regulation with the Wnt/β-catenin signalling. It is thus more probable that the differentiation of digestive epithelium in *Platynereis* will be more similar to *Drosophila* than to vertebrates. The role of Hh might be also ancestral but lost in *Platynereis*. In either case, the differentiation of digestive epithelium would be ensured by another signalling pathway. e. g. the dorsally and laterally located BMP from the ectoderm and later from the mesoderm of the gut wall. Either the mesenchymal and the colonocyte BMP counteracts the Wnt/β-catenin signalling in the vertebrate intestine and points the cells towards a differentiation (Haramis et al., 2004; Hardwick et al., 2004). The Dpp signal from the visceral mesoderm is endowed with a similar capability in the midgut of *Drosophila* [(Nakagoshi, 2005); see the section 7.2.4.3].

The differentiation to digestive epithelium leads to a transcription and production of the digestive enzymes. But why the midgut expresses the hindgut marker *Pdu-Legumain* upon Wnt/β-catenin pathway's inhibition from 5 to 7 dpf?

Pdu-Legumain was reported to be expressed in either the hindgut and the midgut in the 6 dpf stage (Williams et al., 2015) but on the day 7 of development is retained only in the hindgut (Figure 26). At this stage, the Wnt pathway (according to the *Pdu-Axin* and *Pdu-Tcf* expression and the β-catenin protein) has already faded away in this gut compartment (). Hence, *Pdu-Legumain* requires Wnt signalling to be switched off during development in order to continue its expression and becomes inhibited in the midgut by a sustained Wnt/β-catenin signalling activity. This is in contrast required to maintain the expression of the midgut-specific digestive enzymes *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2* and *Pdu-α-Amylase* (at least until the 7 dpf stage). Therefore, the expression of the midgut-specific enzymes diminishes upon an inhibition of Wnt/β-catenin signalling whereas the midgut continues to express the hindgut marker *Pdu-Legumain*.

It is not clear, why the hindgut expression of *Pdu-Legumain* is not abrogated by the activation of Wnt/β-catenin signalling. I assume that it could happen due to the existence of an efficient mechanism which normally inhibits the Wnt/β-catenin signalling in the

hindgut despite it is probably active in the segment addition zone between the last body segment and the pygidium (see the section 11.3.2.1). The best candidate for this function is some kind of feedback regulation which results in the stronger inhibition in the hindgut, the stronger is the Wnt activity in the surrounding tissue. The feedback could be achieved for example by the mutually exclusive interaction between the Wnt/β-catenin and the Hh signalling pathways which I already described above (11.3.1.1).

The wash-out experiment (section 10.4.4.4) makes evident that a pharmacological inhibition of the Wnt/β-catenin pathway between the days 5 and 7 of development does not cause a permanent mis-differentiation of the midgut. The larvae survived, could feed and grew after they were placed from the presence of Wnt/β-catenin inhibitors to a clean natural sea water (Figure 31). Therefore, the treatments were probably performed already after the initial phase of endoderm specification and most of the proliferation (cellularization of the gut) but during the period of differentiation of the endodermal precursors to the midgut digestive epithelium. The endoderm was arrested in a certain phase of its differentiation but present, which is demonstrated by a sustained expression of the endodermal specification TF *Pdu-Nk2.1* either in the midgut and the hindgut at 7 dpf (section 10.4.2.1, Figure 27) or the activity of the endogenous alkaline phosphatase in the entire digestive tract (section 10.4.4.3, Figure 30) even after the inhibition of Wnt/β-catenin signalling. The inhibition hence delayed the onset of expression of differentiation markers and once it was relieved, the activity of Wnt/β-catenin signalling was probably restored and the endoderm could differentate properly.

11.4.2. Endodermal expression of neurogenic genes

11.4.2.1. Dual neural-endodermal nature of some transcription factors

An involvement of neurospecific transcription factors in the development of endoderm is not unusual; in fact, it is very common as demonstrated by the role of *Otx* in the specification of endoderm in sea urchins (Smith et al., 2007; Yuh et al., 2004), *FoxA* and *Nk2* genes in the specification of the ventral neural fates as well as of the endoderm and digestive system (see the section 7.2.3.2 and the chapter 7.2.4 for details), as well as by the specification of enteroendocrine cells by *Pax4*, *Pax6*, *NeuroD1*, *Neurogenin3*, *Nk2.2* and other neurogenic genes (Gehart et al., 2019), a requirement for the *Achaete-scute* bHLH TFs for the midgut development in *Drosophila* (Tepass and Hartenstein, 1995) and numerous other examples. I observed analogous dual neural and endodermal expression in *Platynereis dumerilii* for *Pdu-Otx* and *Pdu-Nk2.1* (chapter 10.4.2) which are expressed in the neuroectoderm of the central episphere as well as in the midgut[2] of nectochaete larvae on the day 7 of development (Figure 27). It has been recently published that the identical expression pattern is displayed also by *FoxA* (Kostyuchenko et al., 2018). It thus seems, that all these genes are parts of an endoderm specification programme that is delayed in the development and is triggered by Wnt/β-catenin signalling during its reactivation in the macromeres and the midgut primordium in *Platynereis* (see the section 11.4.1.3). The shared expression of these transcription factors in the endoderm and the nervous system likely reflects the specification of both tissues by the activity of Wnt/β-catenin signalling and points out to a more general function of Wnt in the specification of tissues that is dependent on the context of the particular germ layer in which these TFs are activated.

11.4.2.2. Neural/endodermal transcription factors show species-specific differences in expression due to heterochrony

Differences in compartmental expression of these endodermal specification genes can be observed even between *Platynereis* and *Capitella teleta*, two species from the same phylum Annelida. The *Otx* gene of *Capitella teleta* (*Ct-Otx*) is first expressed in all gut compartments of the postgastrula and larva; however, the expression of *Ct-Otx* in the midgut is only transitional and is focused to the foregut later in the metatrochophore stage

[2] *Pdu-Nk2.1* is expressed also in the hindgut and hence retains its expression even after the inhibition of the Wnt/β-catenin signalling when the midgut is functionally converted to the hindgut (Figure 27).

(Boyle et al., 2014). This is in contrast with the *Pdu-Otx* gut expression which is first observed in the stomodaeum, i. e. the foregut, and in the peristomium [(Arendt et al., 2001) and the section 10.2.3.1] but becomes confined to the midgut later during the nectochaete stage (section 10.4.2.2). The difference in *Otx* expression between *Platynereis* and *Capitella* can be explained by a heterochrony of development of gut compartments of both species: whereas the midgut development is delayed in *Platynereis* due to high amount of yolk (cf. the section 11.4.1.2), a lower amount of yolk in the eggs of *Capitella* does not inhibit the cleavage of macromeres and the specification of the midgut proceeds normally at approximately the same time as that of other gut compartments.

The expression of *Pdu-Otx* in two cells on both lateral sides of the proctodaeum (Figure 27) does not have a clear counterpart in the *Ct-Otx*, although *Ct-Otx* is transiently expressed in the entire gut of the postgastrulae/early larvae, including the terminal-most cells. Interestingly, the expression in terminal cells of the gut has been found for *Ct-Brachyury*, *Ct-Nk2.1* and *Ct-FoxA* in the *Capitella* metatrochophore stage (Boyle and Seaver, 2008; Boyle et al., 2014). Seemingly same cells express also *Pdu-Brachyury* (Arendt et al., 2001) and *Av-FoxA* in another polychaete *Alitta virens* (Kostyuchenko et al., 2018). It is not clear whether these cells have endodermal or rather neural characteristics. However, in contrast to *Capitella*, I did not detect the expression of *Pdu-Nk2.1* in these proctodeal cells at 7 dpf (Figure 27).

Ct-Nk2.1 is present simultaneously in all parts of the developing gut (Boyle et al., 2014) but I observed *Pdu-Nk2.1* only in the midgut and the hindgut at 7 dpf (Figure 27); nevertheless, it is expressed in the stomodaeum of the *Platynereis* early metatrochophore larvae at 48 hpf (Figure 22). Stomodaeum is the earliest developing gut compartment in *Platynereis* and this expression pattern thus again likely reflects the heterochrony of development between individual parts of the *Platynereis* gut and only transient requirement for the expression of *Pdu-Nk.1* during their specification.

Similarly, *Pdu-FoxA* is expressed transiently in the entire gut (Kostyuchenko et al., 2018), whereas *Ct-FoxA* was not observed in the midgut of *Capitella teleta* but seems to be transiently activated in its cell lineage shortly after gastrulation (Boyle and Seaver, 2008).

Therefore, *Otx*, *Nk2.1* and *FoxA* are expressed only in a certain stage of specification of the midgut endoderm to a digestive epithelium and they diminish here later in development after the differentiation. This is not specific just to annelids as illustrated by a transient midgut and hindgut expression of *Nk2.1* in amphioxus (Venkatesh et al., 1999).

The expression of some neural/endodermal TFs could be retained in secretory endocrine cells that are characteristic by the expression of neural markers (cf. 7.2.4.3). This might be the case of *Nk2.1* whose orthologue *Ct-Nk2.1* is expressed in a scattered pattern reminiscent of enteroendocrine cells in the midgut of *C. teleta* even in the stage when other genes *Ct-Otx* and *Ct-Blimp1* already diminished from the midgut (Boyle et al., 2014). The mammalian *Nk2.1* orthologue is necessary for thyroid development and function (Kusakabe et al., 2006; Lazzaro et al., 1991), while its paralogue *Nk2.2* regulates the specification of enteroendocrine cells (Gross et al., 2015; Gross et al., 2016) and the pancreatic β-cells (Sussel et al., 1998). *Pdu-Nk2.1* may specify for example the MIP-producing cells (Williams et al., 2015). Because *Nk2* genes are Hh targets (Pabst et al., 2000), these cells would be probably Wnt producing cells that receive a Hh signal from the surrounding epithelium with the active Wnt/β-catenin signalling. They might thus represent the stem cell niche of *Platynereis* gut similar to the Paneth cells of the mammalian gut (Sato et al., 2010) that triggers the Wnt/β-catenin pathway and the proliferation of surrounding cells.

11.4.3. *Pdu-Cdx* and the blastoporal legacy of the *Platynereis* gut

Cdx (as suggested by its another name *Caudal*) is generally perceived as a typical caudal gene. *Pdu-Cdx* was reported previously to be active in the hindgut and the posterior cap (ectodermal cells of the pygidium) in the stages from 48 hpf to 6 dpf (de Rosa et al., 2005; Hui et al., 2009), However, I detected its expression also in the ventral midline of the midgut and on the foregut/midgut boundary. During gastrulation, *Pdu-Cdx* is expressed in the posterior blastoporal tissue, lateral blastoporal lips and a subset of prospective stomodaeal cells (de Rosa et al., 2005). Its expression in 7 dpf *Platynereis* larva therefore likely reflects an amphistomous mode of gastrulation in *Platynereis* and indicates that these tissues are derived from the margins of blastopore. It might have been missed in the midline and the foregut-midgut boundary by previous authors due to the delay in the midgut endodermal specification (section 11.4.1.3) which is connected to a reactivation of genes for endodermal specification (11.4.2.2) and apparently entails also a reactivation of endodermal expression of blastoporal genes like *Pdu-Cdx*.

Wnts are expressed (among others) in the pygidium, in the ventral midline and in the stomodaeum of *Platynereis* larvae (Pruitt et al., 2014), similar to what I found for *Pdu-Cdx.* The pharmacological activation of Wnt/β-catenin signalling indicates that *Pdu-Cdx* is a Wnt target gene. Hence it is plausible that the observed *Pdu-Cdx* pattern results from the activation of its transcription by the Wnt/β-catenin signalling activated by nearby sources of Wnt proteins. For example, *Pdu-Cdx* might be transcribed in the ventral midgut midline in response to the production of Wnt proteins by the nearby ventral neuroectodermal midline and/or in an autocrine and paracrine fashion by its own production of Wnts activated by Hh from the even closer axochordal muscle (Lauri et al., 2014). Nevertheless, the Hh signalling should later yield Wnt producing cells themselves unresponsive to Wnt/β-catenin signalling.

The *Cdx* genes of vertebrates are expressed in the posterior half of the primitive streak and later in the neural plate, the mesoderm (i. e. the dorsal tissues) and in the gut up to the foregut/midgut border (Beck et al., 1995; Gaunt et al., 2003), similar to the *Wnt* genes (see the sections 7.2.1.3 and 7.2.3.2). Taking into account the proposed D-V axis inversion in the chordate lineage (Arendt and Nübler-Jung, 1994), the patterns of expression of *Cdx* and *Wnt* genes are conserved across annelids and vertebrates and provide further support to the homology of ventral midline of *Platynereis* (and by extension of protostomes) to the dorsal body midline of vertebrates.

The production of Wnt proteins by the ventral neuroectodermal midline in *Platynereis* is probably also activated by the Hh signal from the underlying axochordal muscle (Lauri et al., 2014) in the same mutually exclusive positive feedback loop which is present on the intersegmental boundaries (section 11.3.1.1). Either the neuroectodermal midline, the axochord and the developing gut express *Pdu-FoxA* (Kostyuchenko et al., 2018; Lauri et al., 2014). The combined expression of *Pdu-Nk2.1* and *Pdu-Nk2.2* also covers both the endoderm and the neuroectoderm of the hyposphere [Figure 27 and (Denes et al., 2007), respectively]. If we imagine the amphistomous gastrulation of *Platynereis*, where the blastoporal lips migrate by the epiboly from the dorsal side around the macromeres of the hyposphere, but not the episphere (section 7.3.5.2), not only the ventral midline and terminal parts of the gut, but also the peristomium descends from the blastopore. Notably, *Pdu-Otx* is present in the peristomium, stomodaeum, the neuroectoderm along the midline and in the developing gut (Figure 21 and Figure 27).

All these genes are thus involved in the specification of endoderm and the central nervous system that is formed around the edge of the blastopore (Tosches and Arendt, 2013). The expression patterns of these genes corresponds to the situation in which they are initially triggered on the vegetal pole of the embryo in concentric domains with different diameters based on their sensitivity to the Wnt/β-catenin and/or Hh signalling. The latter would be triggered by the Wnt signal in the intermediate tier of cells which would give rise to the axochord and separate the central and outer domain. Such concentric Wnt and Hh domains exist on the oral pole of cnidarians [cf. (Matus et al., 2008) and (Wijesena et al., 2017)]. The expression on the outer edge of the blastopore specifies here the medial neuroectoderm while it ceases in the macromeres inside of the blastopore due to a temporal inactivation of Wnt/β-catenin signalling by the yolk. Either Wnt/β-catenin signalling and the expression of these TFs are reactivated in the macromeres after the amount of yolk drops below a critical level. The expression diminishes as other signalling molecules produced with a delay in response to the Wnt/β-catenin signalling eventually overcome the Wnt-induced tissue progenitor programme, the gut endoderm differentiates and the expression of *Wnt* genes becomes limited to stem cell niches.

This model does not apply only to *Platynereis* but a concentric activation of signalling pathways and transcription factors around the blastopore and a successive specification by tiers of regulation with delayed response were well documented in sea urchin (Davidson et al., 2002; Smith et al., 2007) and may represent a hallmark of metazoan development.

11.5. Conclusions

I mapped the expression of several components of the Wnt/β-catenin signalling pathway and manipulated its activity by pharmacological activator and inhibitors in the developing larvae of a marine annelid *Platynereis dumerilii*. I then investigated the gene expression of numerous transcription factors and differentiation markers involved in the development of the central nervous system, in the segmentation of the body and in the development of the digestive system. After a thorough examination, I conclude that the results are in the best agreement with the theoretical scenario inferred from a synthesis of my data on Wnt/β-catenin signalling in *Platynereis* with previously published informations about *Platynereis* and metazoan development. I present it hereafter as "The unified view" because there are many common themes in the function of Wnt/β-catenin signalling in the development of these three tissue/organ systems which are inherently interconnected as parts of a single individual development.

11.5.1. A unified view of the Wnt/β-catenin signalling function in the *Platynereis* development

The signalling activity of Wnt/β-catenin pathway in the developing central nervous system and the digestive tube in *Platynereis* serves as a major source of proliferation and specification of tissue progenitors. That this function need not to be limited to these two organ systems is demonstrated by a general loss of proliferative activity after the inhibition of the Wnt/β-catenin pathway (Figure 29, Figure 30). The proliferation is not connected to overall growth in the lecitotrophic *Platynereis* larvae, but the demands of proliferative cells on nutrition may regulate the distribution of material and energy stored in macromeres. However, the Wnt-dependent proliferation most of all provides a cellular substrate for differentiation and morphogenesis. The morphogenesis is achieved by oriented cell divisions and convergent extension movements that are controlled by non-canonical Wnt signalling pathways, e. g. Wnt/PCP (Steinmetz et al., 2007). Non-canonical *Pdu-Wnt* genes are expressed in patterns analogical to that of the canonical *Pdu-Wnt*s (Pruitt et al., 2014) and hence probably also depend on the activity of the Wnt/β-catenin pathway.

The initial pattern of *Wnt* expression and the Wnt/β-catenin pathway activation is determined by the stereotypic embryonic cleavage with unequal cell divisions [(Schneider and Bowerman, 2007); section 7.3.5.1] and subsequent amphistomous gastrulation (7.3.5.2)

to the lateral episphere and the blastoporal lips. The expression in the ectodermal blastoporal lips is then refined by self-organization due to mutually exclusive non-autonomous positive feedback regulation between the Wnt/β-catenin signalling and the Hedgehog pathway into a segmental pattern of Wnt expression (11.3.1.2), which replicates a subset of the Wnt-dependent gene expression fingerprint from the anterior boundaries of blastoporal lips with the peristomium (11.2.1.3) and/or from the posterior boundaries with the pygidium.

The segmental pattern of Wnt proteins also stimulates the expression of *Engrailed*, the formation of morphological boundaries and (probably via activation of Notch signalling) the production of chaetae and perhaps of other segmental structures. Wnt/β-catenin signalling is active in the posterior segment addition zone between the last body segment and the pygidium, where it activates the expression of *Pdu-Cdx*, stimulates the cell division and the posterior growth. The back-and-forth fluctuations of the anterior Wnt expression boundary are probably responsible for the oscillating gene expression in the growth zone and for the formation of segmental pattern and hence the addition of new segments.

Wnt/β-catenin signalling in the blastoporal lips specifies neural progenitors and after their fusion in the ventral ectodermal midline drives their proliferation in the abutting ectoderm (Demilly et al., 2013). Unlike in vertebrates, the Wnt/β-catenin signalling is active in the medial neuroectodermal plate. The neuroectoderm however displays the same pattern of transcription factors that moreover does not change upon the manipulation of the Wnt/β-catenin signalling pathway. This implies that Wnt/β-catenin signalling is not involved in the medial-lateral patterning of the neuroectoderm in *Platynereis* and perhaps also in other organisms. Instead, it promotes the proliferation and specification of naïve neural progenitors which differentiate according to the cues from the lateral BMP signal and the medial Hh signal, which is produced in the ventromedian axochordal muscle in response to Wnt proteins produced by the ventral midline.

The manipulation of Wnt/β-catenin signalling also does not influence the anterior-posterior patterning of the neuroectoderm by *Pdu-Hox* genes in the trochophore and metatrochophore stages but may be important for their onset. A neurogenic process similar to the one along the ventral midline probably takes place also in the perpendicular segmental domains of Wnt expression where it might generate segmental nerves, and in the lateral episphere, where it is a plausible cause of the expansion of the head lobes and the formation of brain ganglia.

The formation of endodermal epithelium from the macromeres is delayed due to their high amount of yolk which may indirectly inhibit the accumulation of sufficient levels of β-catenin by increasing cell size. Therefore, the endodermal specification programme is postponed and becomes reactivated later in the development after much of the yolk has been re-distributed to other cells in the body. This is documented by the expression of *Pdu-Tcf*, *Pdu-Axin*, accumulation of β-catenin and the activity of the Wnt-responsive transgenic fluorescent reporter. The onset of Wnt activity is connected to a reactivation of the expression of endoderm specification transcription factors, e. g. *Pdu-Otx*, *Pdu-Nk2.1* or *Pdu-FoxA* during the cellularization of the gut and differentiation of the digestive epithelium. Wnt/β-catenin signalling hence probably specifies the proliferating endodermal progenitors in *Platynereis* similar to other bilaterians (sections 7.2.4.1 and 7.2.4.3). Consequently, the inhibition of the Wnt/β-catenin pathway abrogates the expression of the digestive enzymes *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2* and *Pdu-α-Amylase* which are markers of the differentiated digestive epithelium. Instead, the midgut maintains the expression of a hindgut-specific enzyme *Pdu-Legumain* which requires only transitional Wnt activity which occurs during the hindgut development. However, the differentiation is only arrested and continues after the inhibition is released. Non-canonical Wnt pathways co-activated with the canonical pathway in the stomodaeum and proctodaeum may regulate their invaginations and fusion with the midgut in order to form a tripartite gut.

In theory, Wnt signalling in the gut by the positive feedback should co-activate Hedgehog signalling which may become focused to the prospective enteroendocrine Wnt-expressing cells with the expression of neural genes and a stem cell niche function. These cells would activate the Wnt/β-catenin signalling in surrounding cells and stimulate their proliferation and expression of Delta ligands. Some of them would by lateral inhibition become exocrine cells with the activated Wnt/β-catenin signalling and the expression of Delta ligand. Activated Notch signalling would inhibit Wnt pathway in the surrounding cells, destine them to the absorptive fate, break the positive feedback between Wnt and Hh and result in a digestive epithelium with regularly spaced exocrine and endocrine cells. Similar mechanism could function also in the neuroectoderm and may regulate the size of the tissue.

Activation of the same transcription factors (*Pdu-Cdx*, *Pdu-Otx*, *Pdu-Nk2.1*, *Pdu-Nk2.2*, *Pdu-FoxA*) either in the endoderm and the nervous system likely reflects the activation of these genes by the Wnt/β-catenin or Hh signalling in the concentric vegetal tiers of blastomeres. The inner tier of macromeres temporarily deactivates the expression

of these TFs so that they are retained only in the blastoporal lips where they specify the neuroectoderm of the central nervous system. They are later reactivated in the descendants of macromeres which differentiate into the midgut endoderm whereas the intermediate Hh positive tier develops to the muscles including the axochord and the outer Wnt-positive tier gives rise to the ventral neuroectodermal midline.

11.5.2. Homology of the central nervous system

The segmental Wnt expression on the medial ventral peristomium boundary with the cryptic zero segment is a part of a molecular and cell-type signature of *Pdu-Wnt* genes, *Pdu-Pax2/5/8*, *Pdu-En*, *Pdu-Otx* and *Pdu-Gbx*, dopaminergic and serotonergic neurons that homologize this region to the isthmic organizer of the vertebrate brain and the peristomium part of the ventral nerve cords to the midbrain. Unlike in vertebrates, *Pdu-Wnt* genes are expressed posterior to this boundary, which however due to the positive feedback with Hh leads to the activation of Wnt/β-catenin pathway and *Pdu-Otx* expression in the peristomium. Variations on this expression pattern that differ in the *Pdu-Hox* gene in the place of *Pdu-Gbx* are replicated on each intersegmental boundary with the expression of *Pdu-Wnt* genes and include even the ciliary belt present on the prostomium boundary.

Cells close to the anterior prostomium boundary with the episphere express *FoxA* and might represent the homologue of another vertebrate organizer, the zona limitans intrathalamica. This leads me to propose the hypothesis that the insect and vertebrate brain signalling centres are evolutionary descendants of the ciliary belts of ancient planktonic ancestors of Bilateria.

The medial expression of *Pdu-Nk2.1* and *Pdu-Otx* point to a relatedness of the ventral-medial episphere to the ventral thalamus. The apical organ bears a resemblance to the anterior neural ridge and the lateral parts of the episphere could be compared by the expression of *Pdu-Wnt* genes, *Pdu-Pax6*, *Pdu-Six3* and *Pdu-Emx* to the vertebrate telencephalon. Specific parts of the vertebrate brain have been already aligned to their respective homologues in these regions but the homology of other parts remains to be assessed, if there is any. For example, I suggest that the lateral *Pdu-Nk2.1* positive domains may correspond to the medial ganglionic eminences which give rise to the basal ganglia.

11.6. Future perspectives

Platynereis dumerilii proved to be a tractable model system for the study of Wnt/β-catenin signalling in the spiralian development and its evolutionary implications. My results extended our knowledge about the role of Wnt signalling in the development of *Platynereis* and metazoan animals in general. It filled some gaps in proposed homologies, but also brought numerous new hypotheses that could be tested and opened new questions and perspectives for a future research.

Regarding the Wnt/β-catenin signalling pathway itself, nothing is known about Wnt co-receptors LRP5/6/Arrow, Ryk/Derailed or ROR and the Wnt-receptor combinatorial landscape in *Platynereis*, or about its Dishevelled proteins. It would be also beneficial to map the possible activating and repressive forms of Pdu-Tcf, map their expression and identify the isoform expressed in the midgut endoderm.

I did not succeed in attempts to knock out *Pdu-Tcf* or *Pdu-β-catenin* by CRISPR/Cas9 technique (chapter 9.4.2); however, if the role of Wnt/β-catenin signalling is indeed so crucial already in the cleaving embryo, the homozygous mutants could not survive and would die early in development. The heterozygotes could resemble the effect of pharmacological downregulation of Wnt/β-catenin pathway but without the option of time-conditional inhibition which helps to avoid the interference with early developmental functions and their potential contribution thus would be limited. Due to a universal function of Wnt/β-catenin signalling in multiple tissues, the development of a system for conditional knock-out reminiscent of Cre-LoxP system or for ectopic activation would be much more beneficial to study it isolatedly in individual developmental processes. Also a strong stable Wnt-responsive transgene with a rapid response to the pathway's activity would contribute greatly to our knowledge of the sites of activity of Wnt/β-catenin signalling, ideally if it could be crossed with transgenic strains with similar responsive reporter constructs responsive to other signalling pathways and hence could show their interactions.

It turned out that it is necessary to study Wnt/β-catenin signalling in the relation to its regulatory relationships with other signalling pathways, especially Hedgehog. Many of my hypotheses were based on the assumption that the Wnt and Hh signalling regulate each other in a mutually exclusive non-autonomous positive feedback. Although it is in a good congruence with the known facts, a final prove is still missing. It would be necessary to demonstrate that the Hh expression in *Platynereis* is activated by Wnt signalling, be it directly or indirectly via Pdu-En (see the section 7.2.2.2) and that these signalling pathways

preclude the activation of the other in their target cells. It would be also very important to find out whether the Wnt/β-catenin signalling indeed upregulates Delta ligands in *Platynereis* and hence activates Notch in the surrounding cells as it does in vertebrates (7.1.5.2 and 7.2.4.3).

Earlier pharmacological treatments of the Wnt/β-catenin signalling pathway and treatments in different and/or shorter time windows could help to assess whether there is some input of Wnt/β-catenin signalling into the neural patterning after all. An RNAseq of the larvae after pharmacological treatments could help to reveal Wnt target genes but it would not allow to determine which are direct and which indirect or in which cells they are upregulated. Hence only the RNAseq of single cells mapped back to the embryo would make sense. Mapping of target sites in the promoters of the key transcription factors located on the top of developmental executory modules would elucidate the exact inputs of individual signalling pathways to the proliferation, commitment, specification and patterning of tissues while the regulation of components of other signalling pathways could unravel their regulatory relationships. For example, it would be useful to know, what signalling inputs can activate the expression of Wnt genes and which upregulate the inhibitors of Wnt/β-catenin signalling, e. g. Groucho.

A detailed, high resolution expression patterns with *in silico* co-expression could help to assess the proposed homology of the brain signalling centres and more molecular markers should be included in this study. It is possible that the congruence of expression fingerprints will not be absolute, but the differences will be informative of the specific evolutionary changes in each lineage and the origin of these tissues. Consequently, it would require a similar comparative gene expression and embryological study of ciliated larvae and brains from representatives of several other animal phyla to evaluate the theory of the ciliary belts as precursors of the brain signalling centres.

If the model of primary segmentation in *Platynereis* larvae (section 11.3.1.2) is correct, it might be possible by experimentally decreasing the diffusion distance of the Wnt and Hh molecules to increase the number or larval segments. Simpler ways would be a comparative study of related annelid species which have different number of larval body segments and a mathematical modelling.

Cdx gene is involved in the specification of posterior growth zone in arthropods, where it is activated by Wnt signalling. It would be interesting to examine, if the activation of *Pdu-Cdx* by Wnt/β-catenin signalling in *Platynereis* is direct. Even of greater importance would be the finding if Pdu-Cdx also in turn activates the expression of *Pdu-Wnt* genes and

hence might engage in a positive feedback loop with Wnt signalling to maintain the SAZ or whether the positive feedback is rather between the Wnt/β-catenin signalling and *Pdu-Brachyury* or another gene.

By manipulating the nutrient content of the diet and the amount of yolk, it might be possible to study the heterochrony of the gut development and the possible effect of yolk content of β-catenin accumulation.

It would be interesting to see, whether (similar to the neural/endodermal transcription factors) the Wnt/β-catenin signalling is only transiently active during the specification and differentiation of gut epithelium and is also later switched off and by what mechanism. A detailed study of signalling pathways in the adult digestive epithelium could help to answer this question and test the validity of the proposed model for the endoderm differentiation in *Platynereis*, which I predict to be very similar or even identical to that of *Drosophila*.

The study of *Platynereis* development already revealed a lot of surprising facts from the animal evolution. Also the Wnt/β-catenin signalling taught us many valuable lessons from cell and developmental biology. But there still remains a lot hidden. It will take years to investigate and experimentally evaluate all the tempting hypotheses that are now lying in front of us. But it still remains just as important to bring new ones. I cherish a foolish hope that this work might have somewhat contributed to both of these efforts.

12 Acknowledgements

I would like to cordially thank my supervisor RNDr. Zbyněk Kozmik, CSc. for giving me the opportunity to study my favourite signalling pathway in such tremendously interesting organism which is *Platynereis dumerilii* and for offering me the first project on the role of Wnt/β-catenin signalling in the development and evolution of the central nervous system. Also for his sympathetic, friendly attitude, patience and valuable consultations on *Platynereis* experiments and their results. He was pushing me forward in the difficult times when I was accommodating to this challenging but rewarding new emerging animal model.

I also want to thank Bc. Veronika Nosková who tirelessly took care about the feeding, cleaning and breeding of *Platynereis dumerilii* worms in our laboratory cultures. She made the experimental work much easier.

Many thanks come to RNDr. Ondřej Machoň, Ph.D. who generously supplied me with pharmacological inhibitors of Wnt/β-catenin pathway and who also isolated the first fragments of *Pdu-Tcf* that later became a trigger of a project that brought some of my most valuable experimental results.

The colleagues who shared with me the every-day laboratory life created a stimulating scientific and social environment which made the experimental work even more fun and I thank them for that. Last, but not least, I would like to thank the entire Institute of Molecular Genetics of the Czech Academy of Sciences, v. v. i. and especially its microscopic facility for creating excellent conditions to perform research.

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14 Publications

Žídek, R., Machoň, O. and Kozmik, Z. (2018). Wnt/β-catenin signalling is necessary for gut differentiation in a marine annelid, *Platynereis dumerilii*. *EvoDevo* **9**, 25.

Žídek *et al. EvoDevo (2018) 9:14*
https://doi.org/10.1186/s13227-018-0100-7

EvoDevo

RESEARCH

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Wnt/β-catenin signalling is necessary for gut differentiation in a marine annelid, *Platynereis dumerilii*

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Abstract

Background: Wnt/β-catenin (or canonical) signalling pathway activity is necessary and used independently several times for specification of vegetal fate and endoderm, gut differentiation, maintenance of epithelium in adult intestine and the development of gut-derived organs in various vertebrate and non-vertebrate organisms. However, its conservation in later stages of digestive tract development still remains questionable due to the lack of detailed data, mainly from Spiralia.

Results: Here we characterize the *Pdu-Tcf* gene, a *Tcf/LEF* orthologue and a component of Wnt/β-catenin pathway from *Platynereis dumerilii,* a spiralian, marine annelid worm. *Pdu-Tcf* undergoes extensive alternative splicing in the C-terminal region of the gene generating as many as eight mRNA isoforms some of which differ in the presence or absence of a C-clamp domain which suggests a distinct DNA binding activity of individual protein variants. *Pdu-Tcf* is broadly expressed throughout development which is indicative of many functions. One of the most prominent domains that exhibits rather strong *Pdu-Tcf* expression is in the putative precursors of endodermal gut cells which are detected after 72 h post-fertilization (hpf). At day 5 post-fertilization (dpf), *Pdu-Tcf* is expressed in the hindgut and pharynx (foregut), whereas at 7 dpf stage, it is strongly transcribed in the now-cellularized midgut for the first time. In order to gain insight into the role of Wnt/β-catenin signalling, we disrupted its activity using pharmacological inhibitors between day 5 and 7 of development. The inhibition of Wnt/β-catenin signalling led to the loss of midgut marker genes *Subtilisin-1*, *Subtilisin-2*, *α-Amylase* and *Otx* along with a drop in β-catenin protein levels, *Axin* expression in the gut and nearly the complete loss of proliferative activity throughout the body of larva. At the same time, a hindgut marker gene *Legumain* was expanded to the midgut compartment under the same conditions.

Conclusions: Our findings suggest that high Wnt/β-catenin signalling in the midgut might be necessary for proper differentiation of the endoderm to an epithelium capable of secreting digestive enzymes. Together, our data provide evidence for the role of Wnt/β-catenin signalling in gut differentiation in *Platynereis*.

Keywords: Tcf/LEF, C-clamp, HMG, Expression pattern, Protostomia, Polychaeta, Alternative splicing, Proteases, Proliferation, Gut development

Background

Wnt/β-catenin signalling represents one of the most important and intensively studied signalling pathways in metazoan development. Posterior Wnt activity specifies the primary axis in early embryos with regulatory development or orients asymmetrical cell divisions and specifies vegetal cell fates in a similar way in organisms that exhibit a fixed stereotypical development [1]. Vegetal blastomeres usually give rise to the mesoderm and endoderm. For example, Wnt/β-catenin signalling is necessary for both the vegetal fate, midgut and hindgut specification in the sea urchin [2, 3] and is also indispensible for gastrodermal differentiation and maintenance in the sea anemone, *Nematostella vectensis* [4], suggesting

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an ancient role of Wnt/β-catenin signalling in endodermal and gut development. This also illustrates that Wnt/β-catenin can have two or more interconnected or independent roles in the subsequent stages of gut development [5] and homoeostasis [6] where it controls proliferation versus differentiation [7].

One of the most prominent examples is the mammalian intestine. A posterior source of Wnts is involved in the patterning of the primitive gut [5] where it directly activates *Cdx* genes in the hindgut [8] in both vertebrates [9–11] and insects [12]. Additionally, Wnt activity has also been observed on the villi of the developing gut [13]. On the other hand, in the large intestine of adult vertebrates, Wnt signal produced by Paneth cells on the bottom of intestinal crypts is received by neighbouring Lgr5+ stem cells [14]. In response, these cells divide asymmetrically to form progenitors and differentiated cells. Wnt/β-catenin signalling is thus crucial for the renewal of gut epithelium [6]. However, whether a similar role for Wnt/β-catenin signalling also exists in other bilaterian clades remains unclear since much less is known about the role of Wnt/β-catenin signalling in later gut development of protostomes.

In the Wnt/β-catenin pathway, which is active in the early specification of the endoderm and in gut development and maintenance, the transcriptional response is regulated by altering stability and thus the levels of β-catenin protein. In the absence of a Wnt signal, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) in a so-called destruction complex that consists of Axin, adenomatous polyposis coli (APC) and the priming kinase casein kinase-1α (CK-1α). This leads to the ubiquitination of β-catenin by β-TrCP E3 ubiquitin ligase and rapid degradation of β-catenin in proteasomes. With low β-catenin, Tcf family transcription factors in the nucleus bind regulatory regions of target genes and repress transcription [15, 16].

The binding of the Wnt signal protein to an extracellular domain of a Frizzled family receptor ultimately leads to inactivation of the destruction complex. Hence, β-catenin is no longer degraded and can accumulate in the cytoplasm and nucleus, where it binds to a Tcf family transcription factors on the promoters of target genes and provides them with a transactivation domain which allows the activation or derepression of transcription. Levels of β-catenin can thus serve as a read-out of activity of this pathway. Furthermore, Axin is not only a member of the destruction complex, but also a direct Wnt target in mouse [17], human [18, 19] and zebrafish [20]; therefore, the amount of its transcripts corresponds to pathway activity.

There is only one orthologue of the Tcf gene in all Protostomia studied so far, with the exception of Platyhelminthes [16, 21] and Planaria [21] which have three or five *Tcf* genes, respectively. Most vertebrates possess four *Tcf/LEF* genes due to several rounds of whole-genome duplications: *Tcf1* (or *Tcf7*) [22], *LEF1* [23, 24], *Tcf3* (*Tcf7l1*) [25] and *Tcf4* (*Tcf7l2*) [26]. In *Xenopus*, Tcf3 functions as a transcriptional repressor in the absence of a Wnt signal and is replaced at promoters of target genes by Tcf1/β-catenin complex upon Wnt activation [27]. In general, Tcf3 is considered to function mostly as a repressor, Tcf1 and LEF1 as activators, while Tcf4 can function in either capacity, both in the absence or presence of a Wnt signal [28–30].

Tcf proteins contain an N-terminal β-catenin binding domain [31] which is followed by a GBS motif that is recognized by the Groucho co-repressor [32]. On the C terminus, there is a highly conserved HMG box which is followed by a basic tail of amino acids. They together constitute the HMG DNA binding domain (HMG DBD) that recognizes the specific DNA sequence in front of target genes [22, 23]. The basic tail serves as a nuclear localization signal [33] and also helps the HMG domain to bend the bound DNA [34, 35]. All invertebrate (except Planaria and Platyhelminthes) and some vertebrate Tcf genes also encode an auxiliary DNA binding domain that is C-terminal to HMG DBD and contains conserved cysteines, hence called a C-clamp. In vertebrates, only some isoforms of LEF1 and Tcf4 (called E isoforms) possess a C-clamp [36, 37]. C-clamps are thought to help HMG to select, restrict and strengthen binding to their target sites. Some genes, e.g. *Cdx1*, are only activated by isoforms of Tcf that possess a C-clamp [37].

Platynereis dumerilii is a marine polychaete annelid and an emerging spiralian animal model. Its development has been described elsewhere in much greater detail [38, 39]. In summary, *Platynereis* embryos undergo a fixed stereotypical development in which every cell has a defined fate which becomes more restricted with every round of embryonic cleavage. These cleavages are polarized by Wnt/β-catenin pathway activity vegetally in each cell/daughter cell pair [39]. The first three cleavages produce four vegetal macromeres as progenitors of the endoderm [39, 40]. Later, blastoporal lips encapsulate the large macromeres (yolk cells), each containing a lipid droplet. Yolk cells with gut precursors divide in a process called cellularization to form a gut cavity and the larvae start to feed between 5 and 7 days of development [38, 41]. Proliferation continues in the ring of stem cells between the last segment and the pygidium in the segment addition zone [42, 43].

Even though the expression of genes encoding for Wnt proteins, Frizzled receptors and the signal transduction protein Axin has been described in *Platynereis* to some extent, it has been done mostly during earlier stages of

development [44–47]. Surveys of *Platynereis* Wnt genes revealed that it possess 12 out of 13 families of Wnts, with the exception of the "canonical" Wnt3 [45, 48]. They are expressed laterally in the episphere, along the blastopore, in developing and regenerating segments, the ventral midline, the pygidial/proctodeal area and the posterior growth zone [44, 45, 49]. Unfortunately, nothing is known about the expression of *Platynereis* Wnt genes in later larval stages or expression in the gut.

There are four Frizzled receptors of Wnt ligands on signal-receiving cells in *P. dumerilii*, three *Frizzled*-related genes, and two genes which encode for soluble Frizzled receptors [47] that inhibit Wnt/β-catenin signalling. In later development, from 3 to 5 dpf, *Pdu-Fz4* is first expressed in the brain and stomodeum, then later fades out and is more abundant in what appears to be the foregut/midgut and midgut/hindgut borders rather than the mesoderm as reported. *Pdu-Sfrp3/4* is restricted to small expression domains anteriorly to each parapodium at the same stage. However, the expression of other Frizzled-related genes was not examined in these stages.

In this paper, we identified a single *Platynereis dumerilii*'s *Tcf* orthologue, *Pdu-Tcf*, which generates an array of products via alternative splicing. We provide a description of its expression and focus on its function in the developing gut. We also report the presence of other components of Wnt/β-catenin signalling, namely *Pdu-Axin* and β-catenin protein, in the gut. We show that manipulation of the activity of Wnt/β-catenin signalling influences cell proliferation in the developing larva and affects the expression of endodermal markers and gut digestive enzymes, leading to changes in the functional division of the gut. We propose that active Wnt/β-catenin signalling is necessary for cell proliferation and proper differentiation of gut compartments.

Methods

Gene identification and cloning

We searched *P. dumerilii* EST databases with sequences of known *Tcf* homologues found in other organisms and found two contigs, 05083 and 02618, that exhibit a high similarity to the N or C terminus of *Tcf*, respectively. By PCR amplification, we obtained a complete cDNA lacking a C-clamp together with N-terminal probe for in situ hybridization.

The C-clamp (−) isoform, X7, of *Pdu-Tcf* cDNA was amplified by polymerase chain reaction using Long PCR Enzyme Mix (Thermo Fisher Scientific#K0182), forward primer TcfPlatyRT-1 (5′-GGGAGATTTTCATGG CGGATTCA-3′) and the reverse primer TcfPlatyRT-4 (5′-CAGTTAGATCAAGCAGAGGTCAGAAGTAAT ACC-3′) on mixed stage *Platynereis* cDNA as a template. cDNA was synthesized using SuperScript™ II RT (Invitrogen 18064014) and random hexamer primers (Invitrogen 48190-011) following the manufacturer's protocol from mRNA isolated with TRIzol™ Reagent (Invitrogen 15596026) according to manufacturer's instructions. Conditions for PCR were as follows: initial denaturation 95 °C/2 min followed by 30 cycles of 95 °C/20 s denaturation, 61 °C/30 s annealing and at 68 °C/3-min extension, closed by an additional extension period of 10 min. The resulting fragment was then cloned into pGEM T-Easy Vector System (Promega) and sequenced.

The N-terminal fragment of *Pdu-Tcf* utilized for probe synthesis was amplified using Pfu polymerase, together with the TcfPlatyRT1 forward primer with the tail that contained the EcoRI restriction site and the TcfPlaty RT-2 (5′-CTGTACAAGGGATGATGGAACTGG C-3′) + BamHI tail reverse primer. Fragment was then isolated on the gel and the included restriction sites digested by EcoRI and BamHI. Resulting overhangs were used to clone the fragment into the pBlueskript II KS vector and used for probe synthesis.

Sequencing revealed that this *Tcf* possessed a termination codon after the HMG DBD and thus lacked a C-terminal accessory DNA binding C-clamp domain. Since most of the protostomes to which *Platynereis* belongs have only one *Tcf* gene and out of these all possess a C-clamp domain [16, 21], we further searched TSA databases which yielded two more cDNA sequences, GBZT01001652.1 and GBZT01006558.1, from BioProject PRJNA271451 [50] It is of note that the former had a C-clamp and the latter a difference in the beginning of the HMG domain, but otherwise were found to be similar. We designed forward primers that were specific for two different variants of the 5′-HMG exon (5′-TGATGA GAACGAGGTGCAGGA-3′ and 5′-GACCACACACCC AATGATAGCG-3′) and the common reverse primer (5′-TCATAGTGGCGGTGGTTCCA-3′) after the C-clamp using the Primer3 online tool [51, 52]. Different C-terminal isoforms were amplified in two separate PCRs with AccuPrime™ *Pfx* SuperMix (Invitrogen Life Technologies Cat. No. 12344-040), separated by agarose gel electrophoresis, cloned into a pCR™-Blunt vector using a Zero Blunt™ PCR Cloning Kit (Invitrogen, Thermo Fisher Scientific K2750-20) and used for sequencing and probe synthesis.

There is a short 36nt sequence that corresponds to 12 amino acids of β-catenin binding domain after second intron, which is only facultatively included into the transcript. It is present in both available cDNA sequences from the closely related polychaete, *Perinereis nuntia* (NCBI GenBank accession numbers AB701688 and AB701687, [53]), and two *P. dumerilii* TSA sequences but not in another publicly available *P. dumerilii Tcf* sequence

(NCBI GenBank number KT266551, Simon F., unpublished). No such sequence was found in other protostome sequences that we analysed, and an entirely different sequence was observed in deuterostomes. We thus used the N-terminal probe which excluded this variable region to assess the *Pdu-Tcf* expression patterns although we included this region in the phylogenetic analysis.

An *Axin* cDNA fragment was amplified and cloned using a publicly accessible sequence [46] with 5′-AGT TCCTCAATGACTCGGCA-3′ and 5′-CTTCCTGTA ACGTGGGGAGT-3′ forward primer and reverse primers, respectively. The Axin fragment was amplified from mixed stage cDNA by AccuPrime™ *Pfx* SuperMix and cloned into a pCR™-Blunt vector using a Zero Blunt™ PCR Cloning Kit, verified by sequencing, and used to generate digoxigenin-labelled RNA probes.

Templates for probe synthesis of *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2*, *Pdu-α-Amylase* and *Pdu-Legumain* were a gift from Gáspár Jékely's laboratory [41]. Templates for *Pdu-Nk2.1* and *Pdu-Otx* probes were a gift from Detlev Arendt's laboratory [54, 55]. *Pdu-Cdx* clone was obtained as a gift from David K. Ferrier [56].

Protein alignment and molecular phylogeny

The protein alignment was done using BioEdit's [57] ClustalW Multiple alignment and MEGA7's [58] MUSCLE algorithms and improved manually. The GenBank accession numbers of the protein or translated nucleotide sequences used for comparison are as follows: *Perinereis nuntia* (AB701688.1), *Lingula anatina* (XP_013385963.1), *Crassostrea gigas* (XP_019923475.1), *Biomphalaria glabrata* (XP_013060932.1), *Limulus polyphemus* (XP_022255329.1), *Parasteatoda tepidariorum* (XP_021000199.1), *Drosophila melanogaster* (NP_001033798.1), *Tribolium castaneum* (XP_008191151.1), *Strongylocentrotus purpuratus* (NP_999640.1), *Sacoglossus kowalevskii* (XP_006811841.1), *Branchiostoma floridae* (AAZ77711.1), *Danio rerio* (NP_571334.1), *Xenopus laevis* (XP_018082716.1), *Anolis carolinensis* (XP_008112949.1), *Gallus gallus* (XP_015144041.1), *Homo sapiens* (NP_001185456.1). Pdu-Tcf protein sequence is an in silico translation of the longest *Pdu-Tcf* cDNA which we have identified (C-terminal isoform X1, Fig. 2c) obtained by merging the X1 C-terminal sequence with the full-length cDNA of the C-clamp (−) isoform X7 (Fig. 2c).

Molecular phylogeny was determined using MEGA7 software [58]. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model [59]. Initial trees for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among the sites (five categories + G, parameter = 0.8124). All positions with less than 85% site coverage were eliminated. That is, up to 15% alignment gaps, missing data and ambiguous bases were allowed at any position to include the whole HMG domain in the analysis, since two of the sequences (*Perinereis nuntia* and *Limulus polyphaemus*) were truncated. There were a total of 343 positions in the final dataset.

Animal culture and spawnings

Larvae and adult worms were collected from our established *Platynereis* breeding facility at the Institute of Molecular Genetics of the Czech Academy of Sciences in Prague. Mature worms were mated in pairs in natural sea water (NSW) in glass containers. Several minutes after spawning, the worms were discarded, and as soon as fertilized eggs settled to the bottom, most of the volume in the containers was replaced with fresh NSW. The next day, we removed all poorly developing embryos from the bottom of the glass cylinder and kept only the healthy population which used cilia to swim close to the surface of the water column. We kept the developing larvae designated for experiments at 18 °C to ensure proper staging [38].

Sample collection and fixation

Larvae of the desired stage were immobilized by the addition of 1/10–1/3 volume of 4% PFA (from 16% PFA, Electron Microscopy Sciences 50-980-487) in PTw (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl, 0.1% Tween20, pH 7.4) and collected by a pipette into a 2-ml microtube. After sedimentation of the larvae, the solution was discarded and replaced with 2 ml of 4% PFA/PTw. Samples were then incubated at room temperature for 2 h, rocking slowly, followed by three washes with PTw and dehydrated with two washes in 100% MetOH. Subsequently, fixed larvae were stored in 100% MetOH at −20 °C. All solutions used were prepared using diethyl pyrocarbonate (DEPC, Sigma D5758), treated (1:1000) autoclaved deionized water and filtered through a 0.22-µm syringe filters (Merck Millipore SLGS033SS or SLGP033RS) or tissue culture filters (Corning 431097).

Chemical treatment

We concentrated 5 dpf (days post-fertilization) old larvae from a single batch on a fine sieve and collected them using a Pasteur pipette. The whole batch was then divided into four experimental groups, each consisting of 7 ml, in a 6-cm Petri dish. Chemicals used for treatment (dissolved and stored in dimethyl sulphoxide, DMSO,

Sigma) or corresponding amount of DMSO alone as a control were diluted in a volume of NSW to 1 ml in total and mixed with the larvae. We used CHIR99021 as an activator of the Wnt/β-catenin pathway (Biomedica) at 10 μM final concentration, since it works well on earlier (24–48 hpf) stages and higher concentrations often killed the larvae, and inhibitors JW55 (own stock, but available commercially) or IWR-1-endo (Merck Millipore 681669) at a 30 μM concentration. Petri dish with larvae was then incubated at 18 °C for 2 days and collected at 7 dpf stage.

Immunohistochemistry

Immunological staining of the nervous system, cilia, and β-catenin was based on in situ hybridization protocol. Fixation, storage, re-hydration, permeabilization by proteinase K and subsequent washes with PTw were performed in the same manner as the in situ hybridization protocol (see further), followed by blocking in Blocking 1 buffer [39] and incubation with monoclonal anti-acetylated tubulin (1:1000, Sigma T 6793) and anti-β-catenin (1:100, Sigma C2206) primary antibodies in Blocking 1 at 4 °C, overnight, shaking on a nutator.

On the second day, the larvae were washed 3 × 15 and 4 × 30 min in PTw and incubated with Alexa Fluor® 555 goat anti-mouse IgG (H + L) (1:500, Molecular Probes. A21422) and Alexa Fluor® 647 goat anti-rabbit IgG (H + L) (1:500, Molecular Probes 21245) in Blocking 1, in the dark, overnight, shaking on a nutating mixer. Unbound antibodies were removed with several washes of PTw, and the larvae were then transferred via a series of dilutions of gradually increasing concentrations of 2,2′-thiodiethanol in PTw to 97% TDE and stored at 4 °C in the dark.

In situ hybridization on *Platynereis* larvae

Digoxigenin-labelled RNA probes were synthesized using a DIG RNA Labeling Mix (Roche 1277073) from plasmid templates, linearized with the appropriate restriction enzymes, and purified with QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's recommendation, diluted in deionized distilled water, and their concentration measured using the Qubit™ RNA Assay Kit. The probes were then stored at − 80 °C.

Visualization of gene expression by whole mount in situ hybridization was done according to previously published protocols with some minor modifications. Fixed dehydrated larvae stored at − 20 °C in 100% MetOH were rehydrated by washing at least 5 min per wash in decreasing dilution series (75, 50 and 25%, respectively) of MetOH in PTw (DEPC treated, filtered) without rocking at room temperature. After three washes in PTw, the larvae were permeabilized by incubation in freshly prepared 0.1 mg/ml proteinase K (Roche 03115828001) in PTw for 1 min for 24, 48 and 72 hpf larvae, 2–2.5 min for 5 dpf larvae and 2.5–3 min for 7 dpf larvae. Proteinase treatment was stopped using two washes (2.5–3 min) in 2 mg/ml glycine in PTw (prepared in advance and stored in freezer). The larvae were then re-fixed by slow rocking in 4% PFA/PTw for 30 min at room temperature. The fixative was then removed by washing five times in PTw (5 or more minutes each wash), followed by one 10-min wash in hybridization buffer [50% deionized formamide, 0.75 M NaCl, 85 μM sodium citrate, heparin 50 μg/ml, 0.25% Tween20, 1% sodium dodecyl sulphate and 50 μg/ml single-stranded DNA from salmon testes (Sigma D9156) in DEPC-treated deionized water]. After the addition of fresh hybridization buffer, larvae from one sample were divided into several groups for staining of different genes and pre-hybrized for 2–4 h at 63 °C in a thermal block with a cover. After replacement of the pre-hybridization solution with 50 μl of digoxigenin-labelled RNA probes, 2 ng/μl in hybridization buffer (denatured previously for 10 min at 90 °C), the samples were hybridized approximately for 16–18 h overnight in a thermal block at 63 °C.

The following day, probes were collected for reuse and replaced with 250 μl of plain hybridization buffer. After the larvae settled to the bottom of the tubes (10–15 min), another 15-min wash with 250 μl of Hyb-Mix or 0.5 ml of 2× SSCT/50% formamide (Sigma) followed. Subsequently, the samples were washed twice in 2× SSCT/50 formamide for 30 min, twice in 2× SSCT for 15 min and twice in 0.2× SSCT for 30 min. All solutions used from hybridization up to this step were prepared using DEPC-treated autoclaved deionized water and pre-heated to the hybridization temperature of 63 °C The samples were kept in a thermal block at the hybridization temperature throughout this portion of the experiment (even during exchange of solutions). After last SSCT wash, the samples were transferred back to room temperature and washed twice with RT/cold 1× PTw and blocked in 2% w/V Boehringer-Mannheim Blocking Reagent (Roche 11 096 176 001) in maleic acid buffer (0.1 M maleic acid, 0.05 M NaCl, pH 7.5) for 1 h at RT, slowly rocking. The larvae were then incubated with anti-digoxigenin-AP, Fab fragments from sheep (1:4000, Roche 11 093 274 910) and monoclonal anti-acetylated tubulin (1:1000, Sigma T 6793) at 4 °C overnight (the latter was only applied for fluorescent in situ hybridization) while shaking on a nutating mixer.

On the third day, the samples were washed three times with PTw (5–10 min each) and four times in PTw (30 min each). After the last wash, the larvae were stored overnight at 4 °C in PTw. The next morning, the samples were briefly (5–10 min) washed again with PTw.

In the case of fluorescent in situ hybridization, the larvae were washed twice with 100 mM Tris–Cl, pH 8.5, 0.2% Tween20, filtered through a 0.22-µm syringe filters (Merck Millipore), and stained on 4-well or 24-well plate in a Vector® Blue Alkaline Phosphatase Substrate (Vector Laboratories SK-5300) solution which was prepared according to the manufacturer's instructions with 100 mM Tris–Cl, pH 8.5, 0.2% Tween20. A signal was developed in the dark at room temperature while slowly rocking.

For bright-field microscopy, after washing the larvae in PTw, we washed them twice (10 min each wash) in alkaline phosphatase (AP) staining solution without $MgCl_2$ (50 mM Tris–Cl, pH 9.5, 100 mM NaCl, 0.1% Tween20) followed by two washes with the same buffer supplemented with 50 mM $MgCl_2$. All solutions were filtered through a 0.22-µm syringe filter (Merck Millipore). The staining was done in the AP staining solution with $MgCl_2$, 1.65 µl NBT (Roche 11 383 213 001) and 1.65 µl BCIP (Roche 11 383 221 001) per ml. Stock solutions of NBT and BCIP were first centrifuged for 2 min at 14,100 rcf to pellet the precipitated material. A signal was developed in the dark, overnight at 4 °C.

In both cases, the larvae were checked regularly for a developing signal. Staining was stopped by transferring them back to microtubes and washed five times (about 10 min) in PTw.

For fluorescent in situ, larvae were blocked in a Blocking 1 solution [39] consisting of 4% sheep serum, 2 mg/ml bovine serum albumin and 0.1% dimethyl sulphoxide for 1 h at RT, rocking. They were then incubated overnight at 4 °C on nutating mixer in Blocking 1 solution with DAPI (4′,6-diamidino-2-phenylindole, Sigma, 1:1000) and Alexa Fluor® 555 goat anti-mouse IgG (H + L) (1:500, Molecular Probes. A21422) secondary antibody. After three washes (15, 15 and 30 min) in PTw, the larvae were gradually transferred through 10-min washes in a dilution series of 33, 66 and 97% (two times) to 97% 2,2′-thiodiethanol (TDE; Sigma 166782) in PTw and stored at 4 °C in the dark for up to several weeks.

In situ hybridization on adult tails

Adult worms were subjected to starvation for 2 days in order to empty their digestive tracts prior to dissection. They were then immobilized by the addition of 1 M $MgCl_2$ to sea water with worms to the final concentration of 50 mM, which is substantially less than previously used by others [38, 40, 60] but still proved to be sufficient. The last 20–25 segments were cut, and worms were then returned to cultures to regenerate. The tails were fixed in 4% PFA/PTw (DEPC treated, filtered) overnight at room temperature, rocking, washed and dehydrated in the same way as larval samples and stored in methanol at − 20 °C.

Whole mount in situ hybridization was done using the same protocol that was used for larvae with following modifications (as described in [60]): proteinase K treatment was prolonged to 10 min, and the specimens in the pre-hybridization buffer were heated to 80 °C for 30 min prior to hybridization to inactivate endogenous phosphatase activity. The hybridization was done at 63 °C for 90 h. Primary antibodies used were anti-digoxigenin-AP, Fab fragments from sheep (1:4000, Roche 11 093 274 910) and anti-β-catenin (1:100, Sigma C2206). Fluorescent in situ staining was done using Vector® Blue followed by staining with DAPI together with Alexa Fluor® 555 goat anti-mouse IgG (H + L) (1:500, Molecular Probes. A21422) secondary antibody. Stained samples were transferred to and stored in 97% TDE.

Embedding and sectioning

Larvae stained with NBT/BCIP for *Pdu-Tcf* were washed 1× with distilled water, 1× in 70% EtOH for 30 s and 100% ethanol for 1 min. After the ethanol had been removed, it was replaced with 400 µl of Spurr low viscosity embedding resin (Sigma EM0300, prepared according to the manufacturer's instructions) and incubated for at least 20 min while gently rocking. They were then placed into moulds filled with Spurr resin and left for 2 h at room temperature. The larvae were positioned and oriented within the moulds and placed at 72 °C overnight. Blocks were then sectioned to 4-µm thin sections.

EdU labelling of proliferating cells

Chemical treatment with Wnt/β-catenin pathway activator or inhibitors was done from 5 dpf as described. At 6 dpf, 5-ethynyl-2′-deoxyuridine was added to the water to a 20 µM final concentration so it could be incorporated into the DNA of replicating cells until 7 dpf when the larvae would be fixed by 4% PFA/PTw and stored in 100% methanol. Proliferating cells were detected using Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Invitrogen, C10339) according to the manufacturer's instructions, followed by DAPI staining of nuclei overnight. Finally, the larvae were transferred gradually to 97% TDE mounting medium for confocal fluorescence microscopy.

TUNEL detection of cell death

Larvae treated with Wnt/β-catenin pathway activator or inhibitors between days 5 and 7 of development were fixed and stored in 100% methanol. Dead or dying cells were detected using Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Kit (Invitrogen, C10245) and nuclei counterstained with Hoechst 33342 following the kit protocol.

After counterstaining, the larvae were gradually transferred to 97% TDE in PTw.

Microinjections

Fertilized eggs of *P. dumerilii* were washed thoroughly with half a litre of filtered natural sea water (FNSW), treated with 0.1 mg/ml proteinase K in FNSW for 25 s to permeabilize the eggshell and then rinsed quickly with half a litre of FNSW. The zygotes were microinjected with the mixture of 0.4 µg/µl SuperTOPFlash-tdTomato (courtesy of Vladimir Korinek) and 1:5 Fast Green FCF dye. SuperTOPFlash-tdTomato carries the gene for tdTomato fluorescent protein under a promoter with 8 Tcf/LEF binding sites, which makes it a reporter that is responsive to Wnt/β-catenin signalling [61].

Image acquisition, processing and quantification

Stained larvae were placed on a glass slide with three layers of Scotch tape (approximate thickness of each layer was 50 µm) as spacers in 80 µl of 97% TDE as a mounting medium which has the same refractive index as glass and low photobleaching [62]. Immunofluorescent and fluorescent in situ hybridization images were taken using a Leica TCS SP5 AOBS Tandem with LP/-/C HC PL APO 40×/1.30 OIL CS2 or LP/0.14-0.20/D HC PL APO 63×/1.40 OIL objective lenses and with Leica TCS SP8 microscopes with a APO 40×/1.30 OIL CS2 objective as *z*-stacks with a *z*-step of 0.42- and 0.42-µm pixel size resulting in cubic voxels. The appropriate wavelengths for excitation (633 or 635 µm for Vector Blue) and emission detection (720–800 µm for Vector Blue) and *z*-compensation of laser intensity and detector gain to compensate for signal loss with increasing depth in the sample were used. EdU and TUNEL stainings were imaged in Fusion software with 0.45 µm voxel size by Zyla 4.2 PLUS sCMOS camera (Andor) and the Dragonfly 503 spinning-disc confocal system (Andor) mounted on a Leica DMi8 core with HC PL APO 40×/1.30 OIL CS objective lens.

Brightness and contrast were adjusted linearly and uniformly in the same way for all *z*-stacks. Maximum *z*-projections (for β-catenin, EdU and TUNEL stainings) or 3D reconstructions (for in situ hybridization) were done with FIJI software (in the latter case using its 3D Viewer plug-in). Images were cropped and resized in FastStone Image Browser, and the figures were assembled in Adobe Illustrator CS4. Proliferating cells marked by EdU incorporation and dead/dying cells marked by TUNEL staining were counted manually on maximal projections of whole *z*-stacks using the Cell Counter plug-in in FIJI. The differences between treatments were evaluated by a Student's *t*-test.

Bright-field images and composite images of bright field and fluorescence were taken on a Nikon Diaphot 300 inverted microscope with DIC optics by Canon EOS1100D camera and utilizing the Canon EOS Utility software Remote Shooting function. We took a bright-field image from every individual after in situ hybridization and assigned them to categories according to the expression in the gut to quantify the effect of chemical treatment.

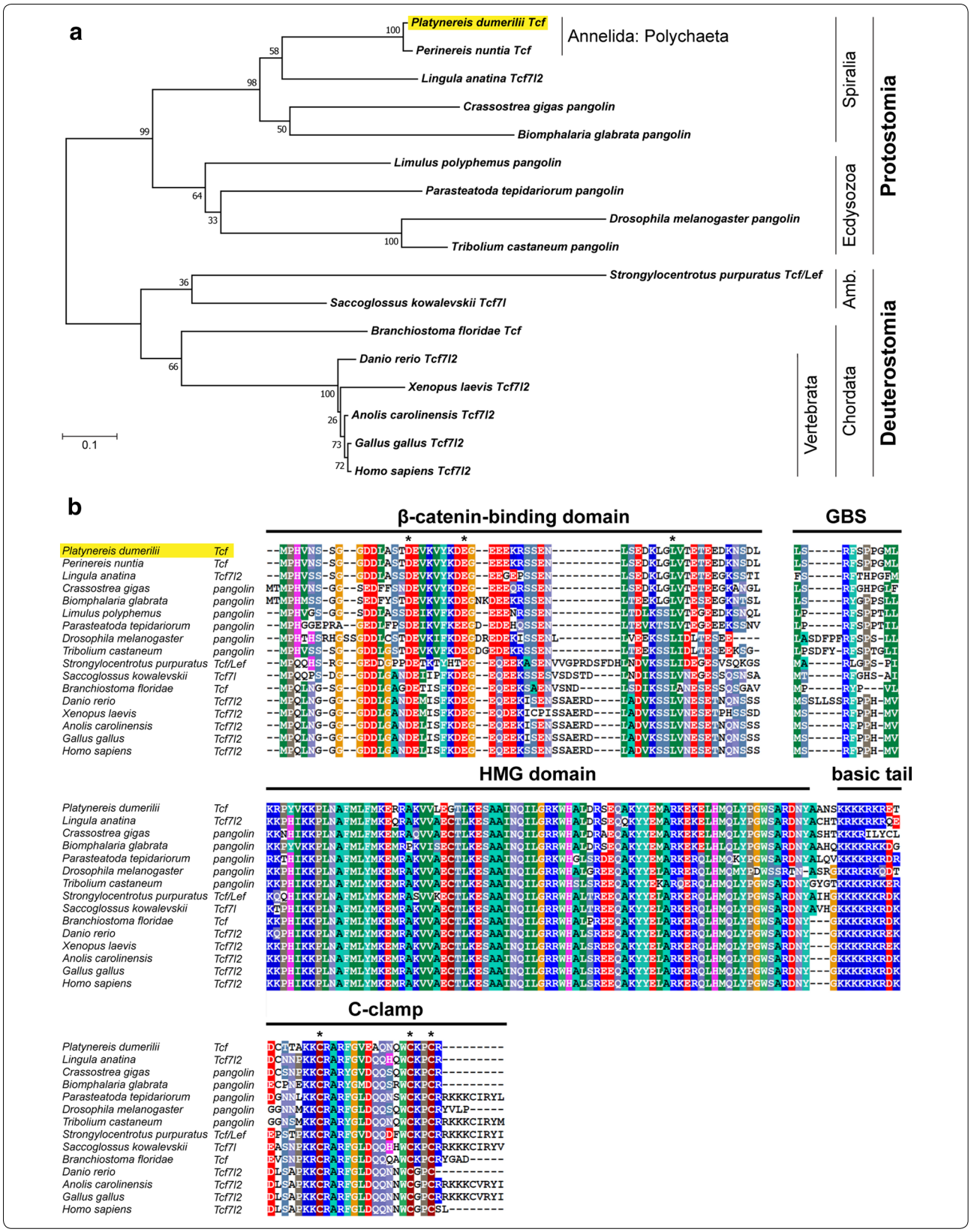
Results

Pdu-Tcf is spliced into multiple isoforms

By BLAST search of EST databases with known sequences from other organisms, we have identified and cloned a homologue of a Tcf/LEF family transcription factor in the marine polychaete annelid *Platynereis dumerilii*. Phylogenetic analysis (Fig. 1a) confirmed that Pdu-Tcf represents a Tcf homologue which is the most similar to that of vertebrate Tcf7l2. Alignment of Pdu-Tcf with selected Tcf protein sequences from other organisms (Fig. 1b) shows that besides the HMG domain and a C-clamp, there is also a conserved N-terminal β-catenin binding domain (amino acids 1–166 as determined by Pfam [63]), GBS Groucho binding sequence and a basic tail, the latter of which immediately follows the HMG domain. *Pdu-Tcf* thus possesses all the necessary functional domains that are present in other organisms and required for the full spectrum of Tcf functions.

We designed forward primers that were specific to both variants of HMG domain and used them with the same reverse primer that was specific to the very end of the C-clamp (+) variant on mixed stage cDNA which was used as a template (Fig. 2a). We observed three products of slightly different sizes for each combination, showing that both HMG variants can combine with C-clamp of the same gene and that there are three variants of C-termini of a different length (Fig. 2b). We used the same combination of primers with genomic DNA to find the source of this variability. Sequencing of the amplified fragment revealed two alternative exons for the HMG domain and only one for C-clamp domain with several potential splice sites. In various C-clamp (+) variants, different splice sites are used, resulting in C-clamp domains of different lengths, which are then freely combined with either of the HMG variants giving rise to six different *Pdu-Tcf* isoforms (marked here as X1–X6, Fig. 2c) with respect to its C terminus.

Moreover, the end of C-clamp (−) Tcf is encoded by an intron of the same gene, directly following the basic tail and containing a cryptic termination codon. When the splice site is skipped and the intron retained, this stop codon prematurely terminates translation, resulting in a shorter protein that does not contain a C-clamp but does have the both β-catenin binding and HMG DNA binding domain, forming a potentially functional transcription


a
Platynereis dumerilii Tcf
Perinereis nuntia Tcf
Annelida: Polychaeta
Lingula anatina Tcf7l2
Crassostrea gigas pangolin
Biomphalaria glabrata pangolin
Limulus polyphemus pangolin
Parasteatoda tepidariorum pangolin
Drosophila melanogaster pangolin
Tribolium castaneum pangolin
Strongylocentrotus purpuratus Tcf/Lef
Saccoglossus kowalevskii Tcf7l
Branchiostoma floridae Tcf
Danio rerio Tcf7l2
Xenopus laevis Tcf7l2
Anolis carolinensis Tcf7l2
Gallus gallus Tcf7l2
Homo sapiens Tcf7l2
100
58
98
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99
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33
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36
66
100
26
73
72
0.1
Spiralia
Ecdysozoa
Protostomia
Amb.
Vertebrata
Chordata
Deuterostomia
b
β-catenin-binding domain
GBS
HMG domain
basic tail
C-clamp

(See figure on previous page.)
Fig. 1 Phylogenetic relationships and conserved protein domains of *Pdu-Tcf*. **a** Phylogenetic analysis of Pdu-Tcf protein (shaded in yellow) sequence with top BLAST hits from selected organisms. The tree reveals that Pdu-Tcf clusters together with another polychaete *Perinereis nuntia* Tcf to the spiralian lineage of bilaterian Tcf sequences and is most similar to Tcf7l2 from the taxa where more *Tcf* genes are present. Protein sequences from taxa where we did not detect a β-catenin binding domain in addition to a HMG DNA binding domain using Pfam [63, 98], though their function as Tcfs was sometimes verified experimentally (e.g. *Caenorhabditis elegans* POP-1), were excluded from the analysis. The higher-order taxa are indicated on the right; Amb. = Ambulacraria. The tree with the highest log likelihood (− 6465.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. **b** Alignment of Pdu-Tcf with Tcf proteins known from other taxa shows the conservation of core domains necessary for Tcf function—N-terminal β-catenin binding domain, GBS—Groucho binding sequence, HMG box DNA binding domain with basic tail and the C-terminal C-clamp accessory DNA binding domain, characterised by the presence of the CRARF(Y) amino acid sequence. Asterisks denote conserved acidic amino acid residues within the β-catenin binding domain and cysteines within the C-clamp domains. Sequences which were incomplete (*P. nuntia* and *L. polyphemus* for HMG domain) or lacked the domain (*Xenopus laevis* for C-clamp) were excluded from the alignment. The extent of highlighted domains corresponds to those used previously by others [16, 21]

factor of yet another isoform (here named X7). Although we observed C-clamp (−) C terminus in combination with only one HMG variant, we cannot rule out that it can also combine with the another one to making a putative isoform, X8. In summary, it appears that, like most protostomes, *Platynereis dumerilii* has only one Tcf gene and a diversity of isoforms is produced by alternative mRNA splicing.

Expression of *Pdu-Tcf*

In order to get an overall view of the *Pdu-Tcf* and Wnt/β-catenin signalling's role in *Platynereis* development, we performed whole mount in situ hybridization with an antisense digoxigenin-labelled probe that was complementary to the N-terminal part of *Pdu-Tcf* mRNA, which is common to and should hence detect all isoforms. We investigated *Pdu-Tcf* expression at 24, 48 and 72 hpf and 5 and 7 dpf larvae as well as adult worms.

At 24 hpf (Fig. 3a, top row), *Pdu-Tcf* was expressed at quite low levels broadly in both episphere (the upper half of the larva above the ciliary belt) and hyposphere (the lower half of the larva below the ciliary belt), except the area around the forming stomodaeum/blastoporus. In the episphere, it was detected slightly more laterally, while it was more abundant in ventrolateral region (neuroectoderm) of the hyposphere.

At 48 hpf (Fig. 3a, second row), the expression of *Pdu-Tcf* was narrowed to more distinct domains, namely ectodermal segmental pattern consistent with the suggested role of Wnt/β-catenin signalling in segment formation

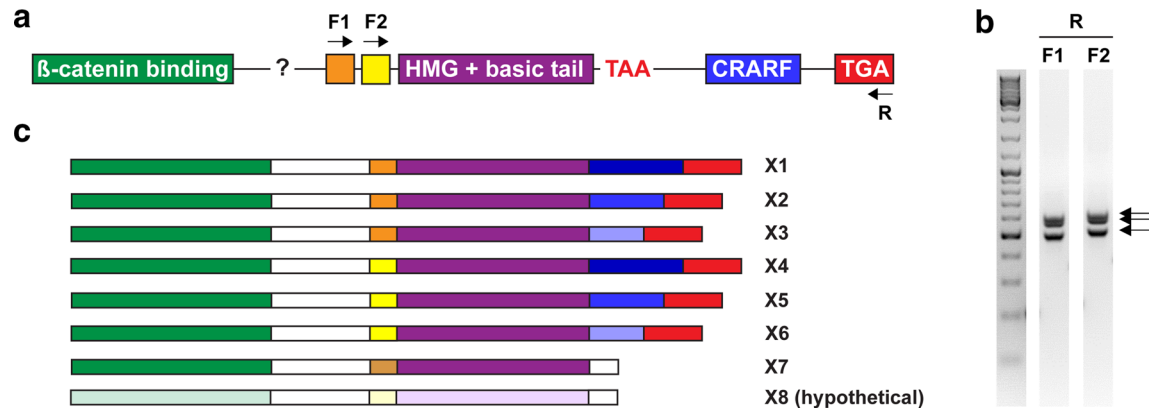

Fig. 2 A single *Platynereis Tcf* gene produces multiple isoforms. **a** Known exon structure of *Pdu-Tcf* C terminus; N-terminal β-catenin binding domain is composed of at least 3 exons; two alternative exons for the beginning of HMG DNA binding domain, a common exon for most of the HMG domain, a premature stop codon within the intron following the HMG exon, alternative splice sites within a single C-clamp (CRARF) exon and a common C-terminal exon. Position of primers used to isolate the individual *Pdu-Tcf* isoforms is indicated. **b** The result of PCR on the cDNA template with the primers indicated in (**a**). Arrows point at three bands corresponding to three different splice variants amplified in each PCR. **c** Graphic representation of identified *Pdu-Tcf* isoforms in relation to their C-termini. We did not isolate the isoform X8, and we saw the isoform X6 only as a PCR product on gel. Note that these are isoforms which are defined only according to their C-termini and do not show the potential diversity of exons and alternative splicing in between the N-terminal β-catenin binding domain-encoding exon and C-terminal exons. Thus, each isoform listed here can actually form two or more variants

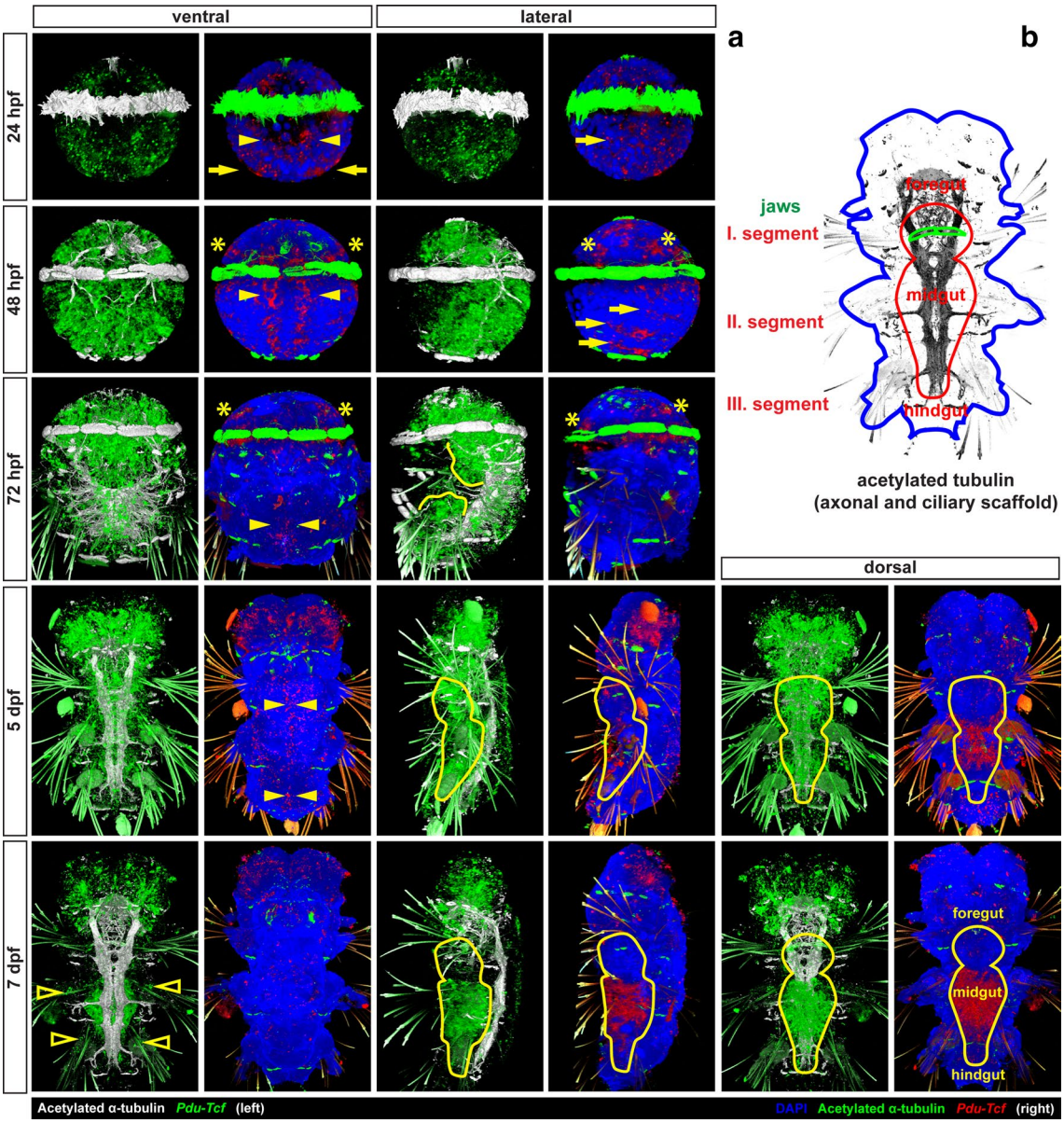

Fig. 3 Expression of *Pdu-Tcf* throughout development of *Platynereis dumerilii*. **a** The expression of *Pdu-Tcf* during development (left: *Tcf* green, acetylated tubulin white; right: *Tcf* red, DAPI blue, acetylated tubulin green). At 24 hpf, *Pdu-Tcf* is broadly expressed at low levels in both the episphere and hyposphere. At 48 hpf in the hyposphere, *Pdu-Tcf* is present in ectodermal cells along the midline (yellow arrowheads) and in the segmental pattern (yellow arrows). It persists in the episphere, e.g. in future larval (ventral) and adult (dorsal) eye regions (yellow asterisks). At 72 hpf stage, *Pdu-Tcf* is expressed mainly in the episphere and stomodeal region, whereas it becomes more scarce in a majority of the hyposphere. This trend continues throughout 5 dpf, where expression mainly restricted to the brain ganglia of the head lobes. At 7 dpf, *Pdu-Tcf* is still expressed in the brain; however, a new strong expression is observed in the midgut and hindgut. There is also a small patch of weaker expression at the base of each parapodium (empty arrowheads). The expression patterns are described in greater detail in the text. Approximate size of a 48 hpf larva is around 130 µm, and all images are to scale. Stage and orientation are indicated; anterior up; in lateral view ventral to the right

[45, 49] and two stripes of ectodermal cells abutting the ventral midline. This domain probably corresponds to axin-expressing proliferating cells which were described by Demilly et al. [46] where it has been documented to respond to the Wnt signal produced by ventral midline. Also the presence of *Pdu-Tcf* in the stomodaeal rosette is quite strong. Ectodermal expression was also present in the apical region, where Wnt/β-catenin signalling has been suggested to regulate apical plate/apical organ development [64]. In the episphere, it can be found in

ventrolateral and dorsolateral patches of ectoderm, the putative larval and adult eye regions, respectively. Moreover, *Pdu-Tcf* has also been observed in the internal segmental mesoderm of chaetal sacs, where also multiple Wnts are present [44] and in putative endodermal cells (Fig. 4, first column).

In 72 hpf larvae (Fig. 3a, third row), the expression persisted in the episphere and the stomodaeal region and it was retained, though weaker, in the two longitudinal stripes abutting ventral midline and diminished in segments. We detected notable delimitated expression domains in two bilaterally symmetrical clusters of cells internally in the larva between the second and third segment and probably continued within the larger central domain located more dorsally (Fig. 4, second column).

Five dpf larvae (Fig. 3a, fourth row) exhibit the strongest expression in brain ganglia. In the stomodaeum, it was expressed in two pairs of bilaterally symmetrical domains, one of which comprised the developing jaws. Weak expression was seen throughout the body, including the ventral midline, spinning glands and developing midgut, with higher expression observed in the hindgut (Fig. 4, third column)

At 7 dpf (Fig. 3a, bottom row), the most prominent expression domain is in the midgut (Fig. 5), while it is still present in the hindgut, as revealed by prolonged staining (Fig. 5a), although in relatively lower level (Fig. 4—fourth column). Besides gut, *Pdu-Tcf* signal still remained high in the brain ganglia, and within sensory organs of the head lobes, it was present, though weaker, in the jaws, while it relatively decreased in the rest of the body. Small patches of cells exhibited the *Tcf* signal at the base of the second and third pair of parapodia and likely correspond to segmental ganglia. High *Pdu-Tcf* expression in the gut was intriguing because Wnt/β-catenin signalling in gut has not been previously reported in *Platynereis* and is also reminiscent of the crucial role of Wnt/β-catenin in gut development and maintenance of other organisms [5–7, 13]. The staining was specific since the sense probe produced no staining (Fig. 5b—right), while levamisole (an inhibitor of endogenous phosphatase, which is present in the larval gut [65]) added to samples during in situ protocol with an antisense probe did not alter staining (not shown).

Next, we analysed the expression of *Pdu-Tcf* in amputated tails of adult worms (Fig. 6). We observed the strongest signal on the luminal side of the gut. *Pdu-Tcf* was also present in the ventral nerve cord, in groups of mesodermal cells, possibly spinning glands and/or excretory system and around the base of chaetae, which might correspond to the signal seen in spinning glands at 5 dpf or segmental ganglia at 7 dpf, respectively (Fig. 3a).

Wnt/β-catenin signalling in the developing gut of *P. dumerilii*

According to the literature, *Platynereis* larvae start to feed on algae between 5 and 7 days of development suggesting that a functional gut is already present [38], and at 6 dpf, larvae possess a gut divided into foregut, midgut and hindgut and express digestive enzymes [41]. Based on our observations, at 5 dpf large macromeres with lipid droplets ("yolk") still obscured the gut cavity and the cellularization occurred between 5 and 7 days of development, since guts of 7 dpf larvae already consist of many cells and possess gut cavity (Fig. 4, right column, Fig. 5a, c).

The presence of several components of the Wnt/β-catenin signalling pathway suggests that Wnt/β-catenin signalling is active in the larval gut. Besides, the expression of *Pdu-Tcf* transcription factor, a relatively high amount of β-catenin, the intracellular transducer of Wnt signal, was detected in the midgut, whereas it was much lower in the hindgut, although we were unable to distinguish nuclear and cytoplasmic staining (Fig. 7a). We also observed higher expression of the putative target gene *Pdu-Axin* in the midgut than hindgut and in the ring of ectodermal cells between the last segment and the pygidium (Fig. 7b).

To further support this evidence, we microinjected a SuperTOPFlash-tdTomato plasmid, which carries a gene for fluorescent protein under the promoter with eight repetitions of Tcf/LEF binding motif. The construct thus acts as a reporter of Wnt/β-catenin pathway activity [61]. We observed a red fluorescent signal in the gut of some transient mosaic transgenic larvae of *Platynereis* at 7 dpf (Fig. 7d), mainly in macromeres, where β-catenin is known to be stabilized from early development [39]. Thus, Wnt/β-catenin signalling is indeed active in the developing gut of *P. dumerilii* larvae.

Pharmacological manipulation of Wnt/β-catenin pathway

To gain insights into the function of Wnt/β-catenin signalling in the *Platynereis* gut, we decided to chemically activate or inhibit the Wnt/β-catenin pathway in developing larvae from 5 dpf, when the *Pdu-Tcf* was still low in the midgut, to 7 dpf, when we observed the highest expression of *Pdu-Tcf*. To achieve this, we added chemical inhibitors or an activator of Wnt/β-catenin signalling to the sea water containing larvae. After chemical treatment, we fixed the larvae and observed differences in expression of gut-specific markers by in situ hybridization. Since after each chemical treatment, we often observed an array of phenotypes with different intensity and sometimes even a slightly variating pattern of staining, we attempted to quantify the results to measure the differences between the treatments by assigning every

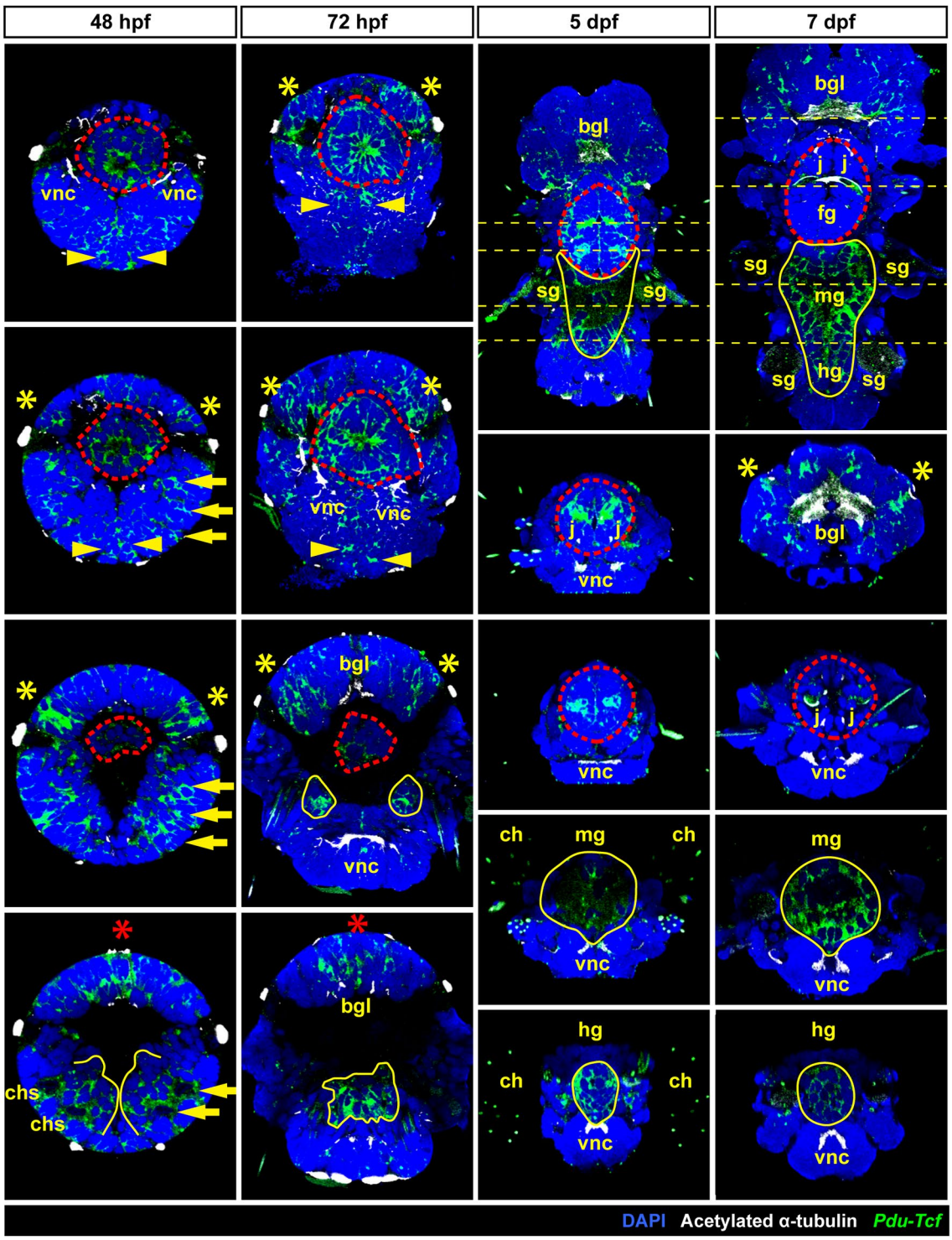

Fig. 4 Analysis of *Pdu-Tcf* expression. Images show virtual orthogonal sections through confocal fluorescent *z*-stacks of *Pdu-Tcf* in situ hybridization staining to illustrate inner features and expression domains. The stages are indicated; 48 and 72 hpf—coronal sections from ventral to dorsal; 5 and 7 dpf, top—coronal sections with the positions of transverse sections indicated; 5 and 7 dpf, from top to bottom—consecutive transverse sections from anterior to posterior as indicated by a yellow dashed line on the coronal sections. The approximate size of a 48 hpf larva is around 130 µm, and other images are to scale. Red asterisk—apical organ, red dashed line—stomodaeal rosette/pharynx (foregut), yellow arrow—segmental expression in chaetal sacs, yellow asterisk—eye-forming region, yellow dashed line—position of transverse sections, yellow line—midgut + hindgut or putative gut tissue. *bgl* brain ganglia, *ch* chaetae, *chs* chaetal sacs, *fg* foregut, *hg* hindgut, *j* jaws, *mg* midgut, *sg* spinning glands, *vnc* ventral nerve cord(s)

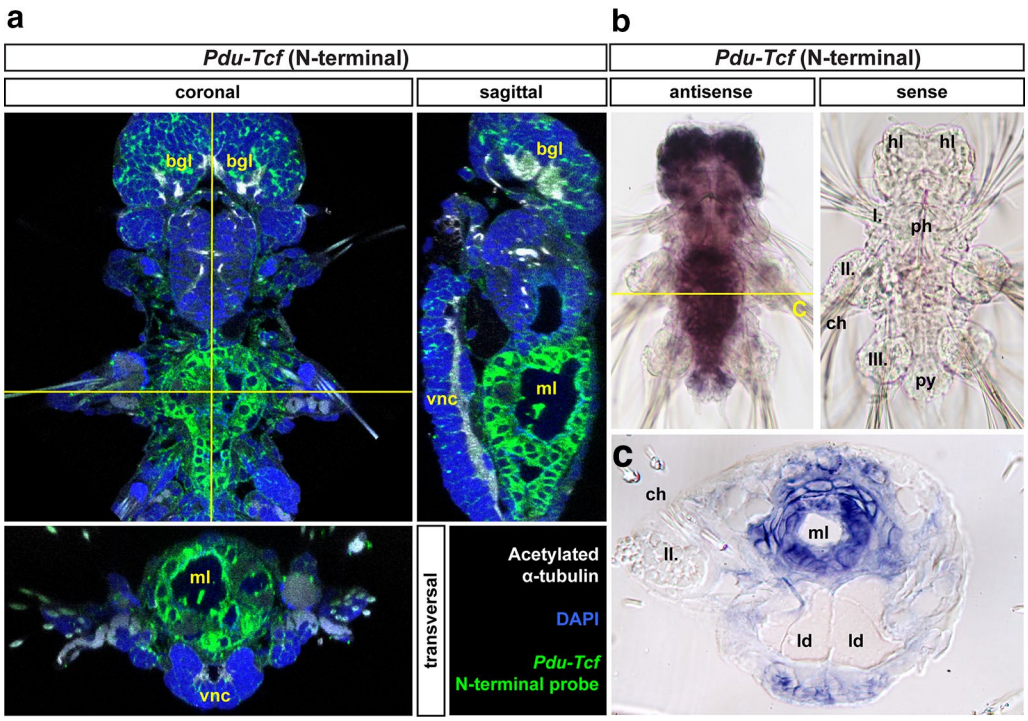

Fig. 5 *Pdu-Tcf* is specifically detected in the gut of 7 dpf larva by N-terminal probe. **a** Virtual orthogonal sections through a confocal *z*-stack of a 7 dpf *Platynereis* larva after prolonged staining of fluorescent in situ hybridizaton with a probe recognizing the N-terminal part of *Pdu-Tcf* show the expression of this gene in the midgut and hindgut. **b** *Pdu-Tcf* N-terminal antisense (left) and sense (right) probe NBT/BCIP stainings show that the antisense probe specifically detects *Pdu-Tcf* in the brain and in the gut. Also treatment with levamisole did not abolish the antisense probe staining demonstrating that the observed signal was not due to persisting endogenous alkaline phosphatase activity (which was thus successfully inactivated by hybridization temperature). The same fact is also demonstrated by the lack of staining with sense probe (right) or after hybridization without a probe (not shown). The absence of signal with sense probe shows that the observed signal is not due to unspecific binding of digoxigenin-labelled probe. **c** Physical coronal section through the body of 7 dpf larva after in situ hybridization with antisense probe against N terminus of *Pdu-Tcf* confirms its presence in the gut. The section is approximately on the level of the second segment, i.e. through midgut. I., II., III.—first, second and third body segments' parapodia, *bgl* brain ganglia, *hl* head lobes, *ld* lipid droplets, *ml* midgut lumen, *ph* pharynx (foregut), *py* pygidium, *vnc* ventral nerve cord. For schematics of 7 dpf larval gut morphology, see Fig. 2

individual to categories according to their degree of marker expression. To ensure that the observed differences were due to the altered activity of Wnt/β-catenin signalling, we verified the efficacy of treatment by whole mount immunodetection of β-catenin (Fig. 7a) and by in situ hybridization of *Pdu-Axin* mRNA—Fig. 7b, c).

It has been previously shown that CHIR99021 activates Wnt/β-catenin signalling via the inhibition of GSK-3β, a part of the destruction complex, thus stabilizing β-catenin [66, 67]. JW55 has been shown previously to inhibit Wnt/β-catenin signalling in a cell reporter, gene expression and *Xenopus* double axis systems [68]. IWR-1-endo was previously demonstrated as a potent inhibitor of Wnt/β-catenin pathway [69] and has been used to inhibit Wnt/β-catenin signalling in *Platynereis* [46]. Both JW55 and IWR-1 function through the inhibition of tankyrase [68, 70]. We decided to use both inhibitors at a 30 μM final concentration, although 40 μM was used previously by others [46]. In our hands at 5–7 dpf stage, we saw an effect even with the 30 μM concentration; on the other hand, the higher concentration may have been lethal for the larvae. Our results suggest that IWR-1-endo was more potent and reliable Wnt inhibitor than JW55 and that it lowered β-catenin levels in the gut (Fig. 7a). On the other hand, JW55 seemed not to work as well on some batches; however when it did, both inhibitors yielded consistent results. Interestingly, none of the inhibitors caused the complete loss of expression of a putative Wnt target gene *Pdu-Axin*. This might be attributed to the possibility that *Pdu-Axin* is only partially regulated by Wnt/β-catenin signalling (for details, see "Discussion" section). Although tankyrase inhibitors act through the stabilization of Axin, this happens on the protein level and should not affect mRNA. We were interested to see whether *Pdu-Tcf* itself was a target of Wnt/β-catenin. There appear to be fewer *Pdu-Tcf*

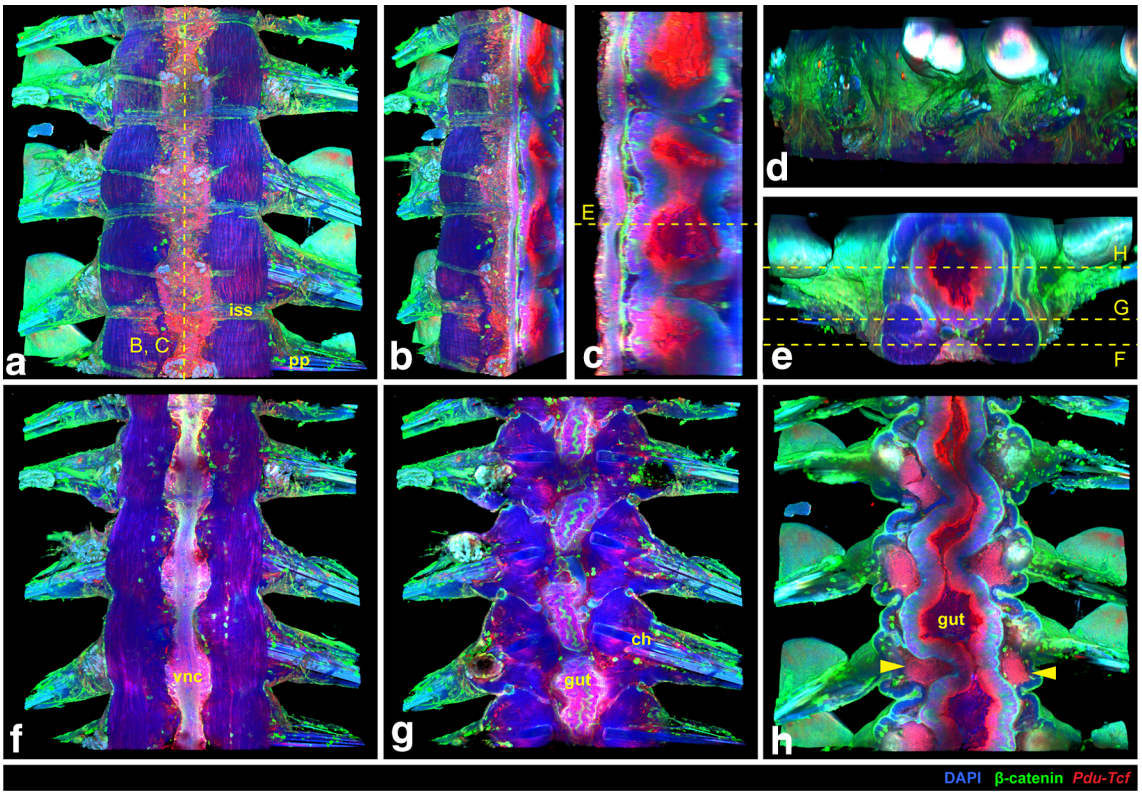

Fig. 6 Expression of *Pdu-Tcf* in the adult worm. **a** 3D reconstruction of a confocal *z*-stack of several adult *Platynereis* trunk to tail segments showing the expression of *Pdu-Tcf* by fluorescent in situ hybridization (red) together with DAPI (blue) and β-catenin immunostaining (green) to visualize cell nuclei and surface; ventral view, anterior up. Sagittal section of the *z*-stack from (**a**) through the midline from ventrolateral (**b**) or lateral (**c**) view, ventral to the left, anterior up. Strong *Pdu-Tcf* expression signal in the intestine and constrictions of the gut between segments are apparent. **d** Lateral view of the same *z*-stack; ventral down, anterior to the left. **e** Transverse section through the middle of segment marked in (**c**) with the planes of coronal sections in (**f**–**h**) indicated. **f** Coronal section of the *z*-stack from (**a**) on the level of the ventral nerve cord (VNC), as indicated in (**e**). Some in situ signal is present in the VNC and weakly in muscles. **g** Another coronal section deeper in the body through the intestinal wall. In situ signal can be seen in the gut and in few cells around the base of chatae. β-catenin staining is strongest on the surface of the body and the gut epithelium. **h** Coronal section through the middle of the gut demonstrates strong *Pdu-Tcf* intestinal expression, especially on the luminal side, and in the intersegmental clusters of mesodermal cells (marked by yellow arrowheads), most probably the excretory system. *ch* chaetae, *iss* intersegmental septum, *pp* parapodia, *vnc* ventral nerve cord

transcripts upon Wnt/β-catenin inhibition and more upon activation in the gut, which suggests a positive feedback loop, whereas the opposite is the case for the rest of the body. However, these conclusions are based on a single observation and the effect was not quantified.

Wnt/β-catenin signalling regulates proliferation

Wnt/β-catenin signalling in general induces cell proliferation [71], and in vertebrate gut, Wnt activity at the base of crypts maintains cells in a proliferative state. Therefore, we were curious, whether a similar effect on cell proliferation in *Platynereis dumerilii* could be observed as well. We performed chemical activation and inhibition treatments from 5 to 7 dpf as described above, but this time we added the nucleotide analogue 5-ethynyl-2′-deoxyuridine (EdU), which is incorporated into replicating DNA in vivo, to the water containing 6 dpf larvae. EdU contains an ethynyl group that after activation is later labelled with a fluorescent dye to visualize proliferating cells [72].

In *Platynereis* at 7 dpf, most of the proliferating cells were located in the frontal part of the head and in the proliferative zone between the last segment and pygidium, surrounding the hindgut (Fig. 8a). Some cells could be seen in pygidium itself at the base of anal cirri and around anus (potentially identical to *Pdu-Otx*-expressing cells, see further), at the base of second and third pair of parapodia, the jaws and on the foregut/midgut and midgut/hindgut borders. Only few proliferating cells were seen on the ventral side of midgut.

Overall, there were significantly less proliferating cells throughout the body of IWR-1-endo treated larvae, whereas the number of proliferating cells in the presence of Wnt activator CHIR99021 was not changed (Fig. 8c).

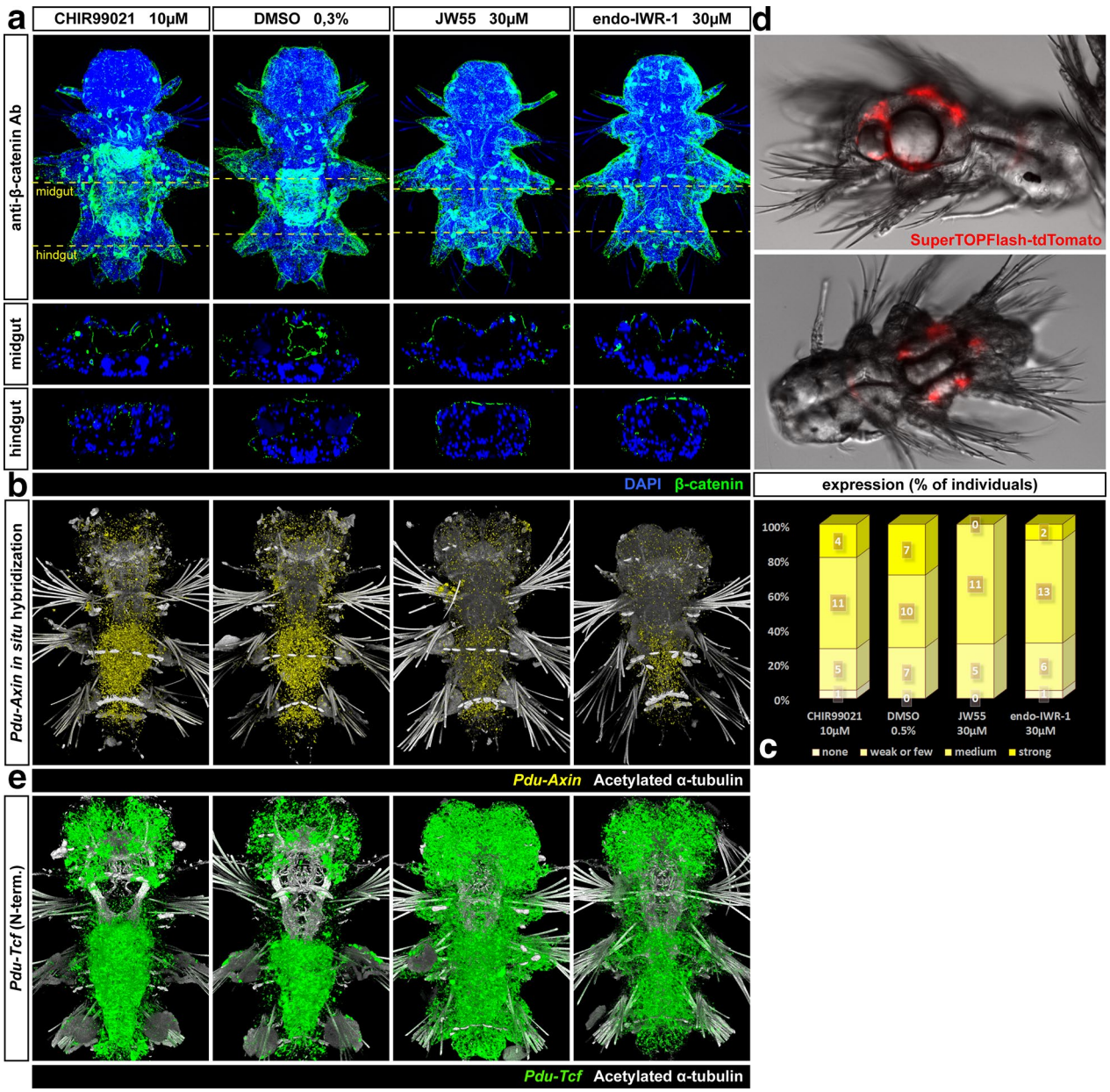

Fig. 7 Wnt/β-catenin pathway is active in the larval gut and is affected by chemical manipulation. **a** Maximal projections and orthogonal virtual sections on the level of midgut and hindgut of a fluorescent confocal *z*-stacks of β-catenin protein immunostaining (green) on 7 dpf larvae done to confirm the chemical treatment's efficacy. The larvae shown here come from the same batch as those used for in situ hybridization in Fig. 9. High levels of β-catenin were observed especially in the midgut. While the activation by CHIR99021 did not cause any dramatic increase in β-catenin levels, the inhibition of Wnt/β-catenin signalling by either of the inhibitors, JW55 or IWR-1-endo led to the complete absence of such high levels of β-catenin in the gut. **b** Fluorescent in situ hybridization of a putative Wnt target gene, *Pdu-Axin* (yellow) and quantification of the phenotype classes (**c**) shows no effect for the activator (CHIR99021) and mild effects of both Wnt/β-catenin inhibitors on axin expression. These larvae come from the same batch as those used for in situ hybridization in Fig. 10. **d** The fluorescent signal from the microinjected Wnt reporter construct SuperTOPFlash-tdTomato with 8 Tcf/LEF binding sites can be observed in the endoderm of 7 dpf transient transgenic larvae. This indicates that the Wnt/β-catenin signalling is active in the gut. **e** The effect of chemical manipulation of Wnt/β-catenin signalling on the expression of *Pdu-Tcf*

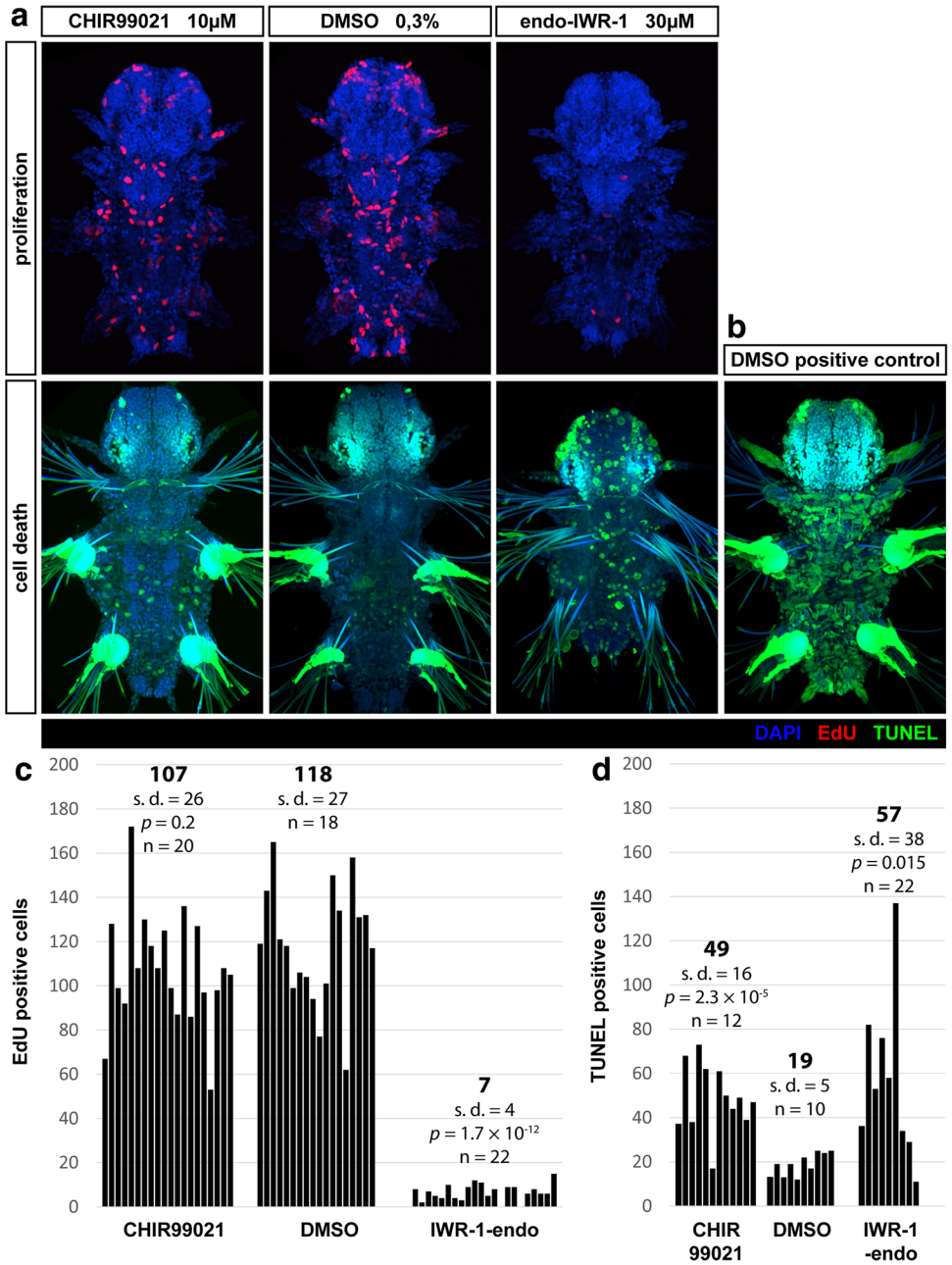

Fig. 8 Wnt/β-catenin signalling is necessary for cell proliferation and survival. **a** Proliferating cells labelled by incorporation of 5-ethynyl-2′-deoxyuridine (EdU, red) from 6 to 7 dpf and counterstained with DAPI (blue) to mark cell nuclei. Images are maximal projections of the whole fluorescent confocal *z*-stacks. Representative individuals are shown. **b** TUNEL staining of cell death (green), counterstained with Hoechst dye (blue) to mark nuclei. **c** Number of EdU positive proliferating cells in the whole larvae as counted manually on maximal projections of fluorescent *z*-stacks as those shown in (**a**). Averages are indicated in bold, *n*—number of individuals analysed per each experimental group, *p*—*p* value of a standard two tailed unpaired Student's t test with unequal variance, *s.d.*—standard deviation. There are significantly less proliferating cells when IWR-1-endo is present. With the activator of Wnt/β-catenin signalling CHIR99021, there is no significant difference in the number of proliferating cells throughout the body. **d** Quantification of cell death performed in the same way as for proliferation, except only cells from the trunk up to midgut/pharynx boundary (or up to first pair of parapodia) were counted. There are significantly more dead or dying cells in groups treated with CHIR99021 or IWR-1-endo compared to control group which was treated with DMSO

Thus, it seems that active Wnt/β-catenin signalling is required for cell proliferation.

We also checked for cell death by TUNEL staining of fragmented DNA. There were significantly more dead or dying cells in the larvae treated with CHIR99021 or IWR-1-endo. However, this test does not discriminate between apoptosis and other forms of cell death. Since most dead cells are present on the surface of the body, which is in direct contact with the environment, and the dead cells are seen in the presence of either activator or inhibitor, the cell death cannot account for the observed changes in gene expression inside the gut which are different for each chemical. Moreover, expression of some gut marker genes shows that a morphologically normal gut was present in all experimental groups.

Downregulation of Wnt/β-catenin pathway converts midgut to hindgut

Platynereis gut consists of three major parts, a foregut, a midgut and a hindgut. To visualize the effect of chemical manipulation of the Wnt/β-catenin pathway on gut differentiation, we used the expression of several digestive enzymes that have been previously described to be specific to different *Platynereis* gut compartments [41]: extracellular peptidases *Pdu-Subtilisin-1* and *Pdu-Subtilisin-2*, a polysaccharide digesting enzyme *Pdu-α-Amylase* and a precursor of an intracellular digestive protease *Pdu-Legumain*.

Pdu-Subtilisin-1 showed only faint expression in the hindgut and was primarily expressed in the midgut (Fig. 9, top row), similar to *Pdu-Subtilisin-2* (Fig. 9, second row) and *Pdu-α-Amylase* (Fig. 9, third row). All three of these genes thus exhibited predominantly midgut expression (in the latter two consistently with the pattern described by Williams et al. [41]). We observed much stronger expression of *Pdu-Legumain* protease precursor in the hindgut than in the midgut, making *Pdu-Legumain* a useful hindgut marker gene. The results of quantification are summarized in Fig. 9b. Genes expressed in the midgut, but not in the hindgut at 7 dpf (*Pdu-Subtilisin-1*, *Pdu-Subtilisin-2*, *Pdu-α-Amylase*) were expressed at lower levels or diminished in the midgut upon Wnt/β-catenin inhibition (Fig. 9, top, second and third rows), while the hindgut-specific gene *Pdu-Legumain* (Fig. 9, bottom row) expanded from the hindgut to midgut and even outside gut to nephridia (compare with [65]) under the same conditions. The midgut thus obtains hindgut-like characteristics when the Wnt/β-catenin signalling is inhibited. Activation of the Wnt/β-catenin pathway by CHIR99021 had no major effects on gene expression, which might reflect that Wnt/β-catenin signalling was already active in the midgut, where it is required, but not sufficient, to trigger midgut fate.

We also looked at the expression of two endomesodermal genes, *Pdu-Nk2.1* and *Pdu-Otx*. They are typical for the neuroectoderm early in its development [73–76], but *Otx* has also been documented in stomodaeum [55, 75]. However, both genes are known to be important regulators of endomesodermal and gut development of Bilateria, e.g. starfish [77], and even Cnidaria [78]. In addition, both were also found in the developing gut of other annelids—*Capitella teleta* [79] or (in the case of *Lox10* gene, a plausible *nk2* homologue) the leech *Hellobdela triseralis* [80]. In 7 dpf *Platynereis*, *Pdu-Nk2.1* (Fig. 10, top row) was expressed in a large domain in the middle of head between two stems of axons protruding anteriorly from the brain and also in two smaller domains on sides of the head, laterally from these axonal bundles in the eye region. In the gut, similarly high levels were observed in both midgut and hindgut, which is consistent with what has been published for *Capitella* [79]. *Pdu-Otx* transcripts were found throughout the head, more abundant in the domains that were medially adjacent to the forementioned axonal bundles, the small ectodermal patches anterior from these axons, and on the posterior–lateral sides of the head. In the digestive tract, *Pdu-Otx* (Fig. 10, bottom row) was expressed in the jaws, in the midgut, but not hindgut, and strongly in two cells surrounding the anus. It diminishes from the midgut upon the inhibition of Wnt/β-catenin pathway by IWR-1-endo, similar to midgut-specific enzymes. The loss of *Pdu-Otx* expression from the two anal cells in the presence of either activator or inhibitor suggests that these cells require a precisely regulated activity of the Wnt/β-catenin signalling to activate *Pdu-Otx*. *Pdu-Nk2.1*, which is normally expressed in both the midgut and hindgut, does not change its expression pattern upon either activation or inhibition.

The role of the *Cdx* gene in hindgut formation has been well documented in vertebrates (reviewed in [5]), sea urchins [81], ascidians [82], *Drosophila* [12] and other organisms. It is directly activated by Wnt/β-catenin signalling via Tcf4 [8], or it can conversely trigger Wnt expression as seen in the sea urchin [81]. *Pdu-Cdx* is also expressed in the hindgut of *Platynereis* [43, 56] and *Capitella* [83]. We used in situ hybridization of a *Pdu-Cdx* probe to detect its expression after chemical treatment of Wnt/β-catenin signalling. In controls, *Pdu-Cdx* was expressed in the hindgut and the foregut/midgut boundary, with these two domains connected by a weaker expression on the ventral side of the midgut (Fig. 11a), close to sources of Wnt and consistent with the notion that *Cdx* genes require a high Wnt signal for activation. Interestingly, *Pdu-Cdx* expands from the ventral side through the entire midgut upon Wnt activation in some individuals but was expressed in a normal pattern, though at somewhat lower levels, when the pathway is

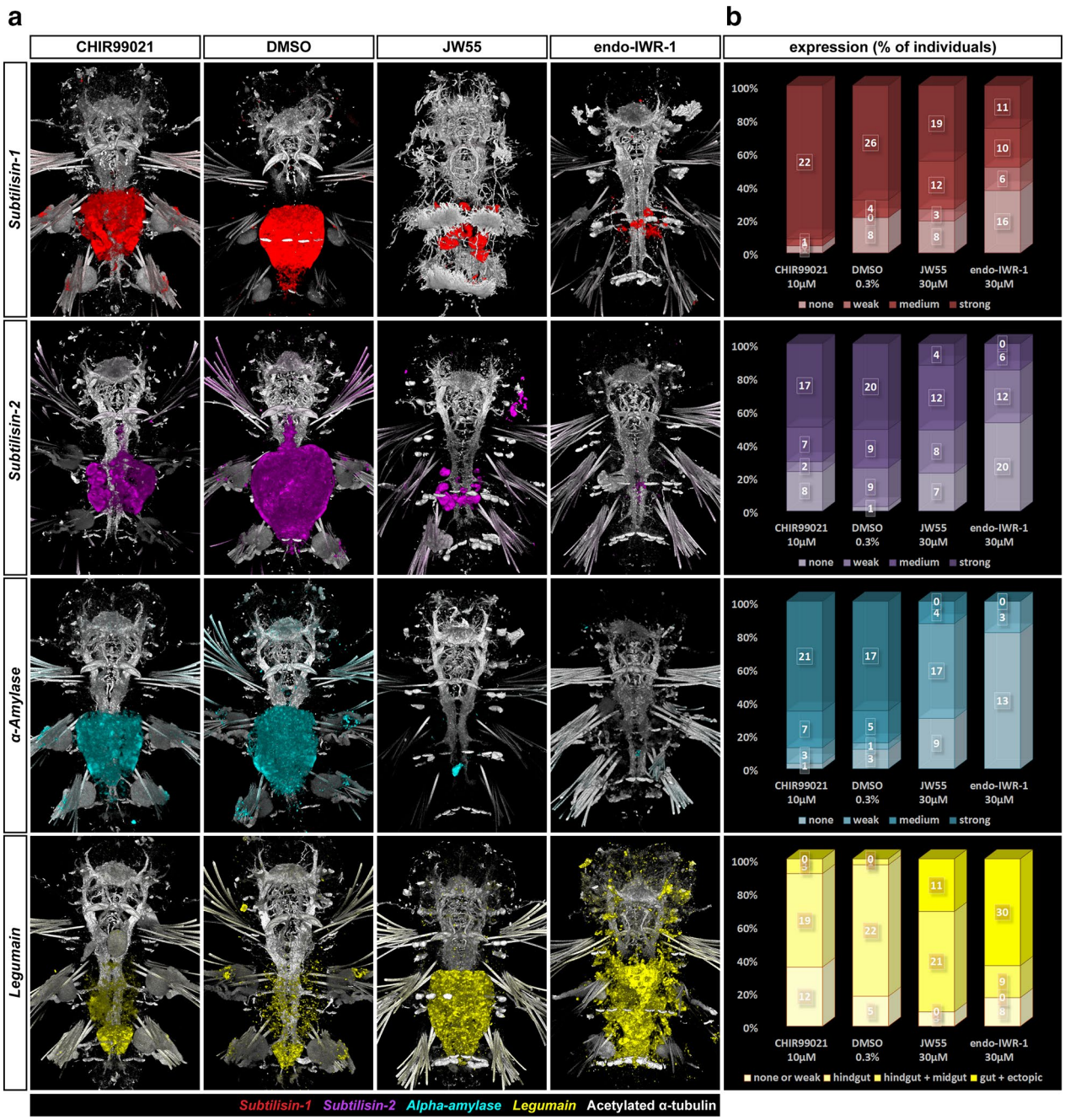

Fig. 9 Inhibition of Wnt/β-catenin signalling converts the midgut to hindgut. **a** Fluorescent in situ hybridization of gut marker genes. **b** Quantification of the effect of activation/inhibition of Wnt/β-catenin pathway on the expression of these genes by assignment of all individuals to phenotypic classes. *Pdu-Subtilisin-1* (first row, red), *Pdu-Subtilisin-2* (second row, magenta), *Pdu-α-Amylase* (third row, cyan) = midgut marker genes, the darker the colour in the graph, the higher the expression. All three midgut marker genes show remarkably lower or no expression when Wnt/β-catenin signalling is inhibited (JW55 or IWR-1-endo). No pronounced effect was observed for activation of the pathway (CHIR99021). *Pdu-Legumain* (fourth row, yellow) = hindgut marker gene is normally highly expressed in the hindgut but only in very low levels in the midgut, which is also the case in the presence of activator, but it expands to midgut upon Wnt/β-catenin inhibition. Moreover, stronger inhibition (IWR-1-endo) causes it to be expressed outside the digestive tract in nephridia and other cells

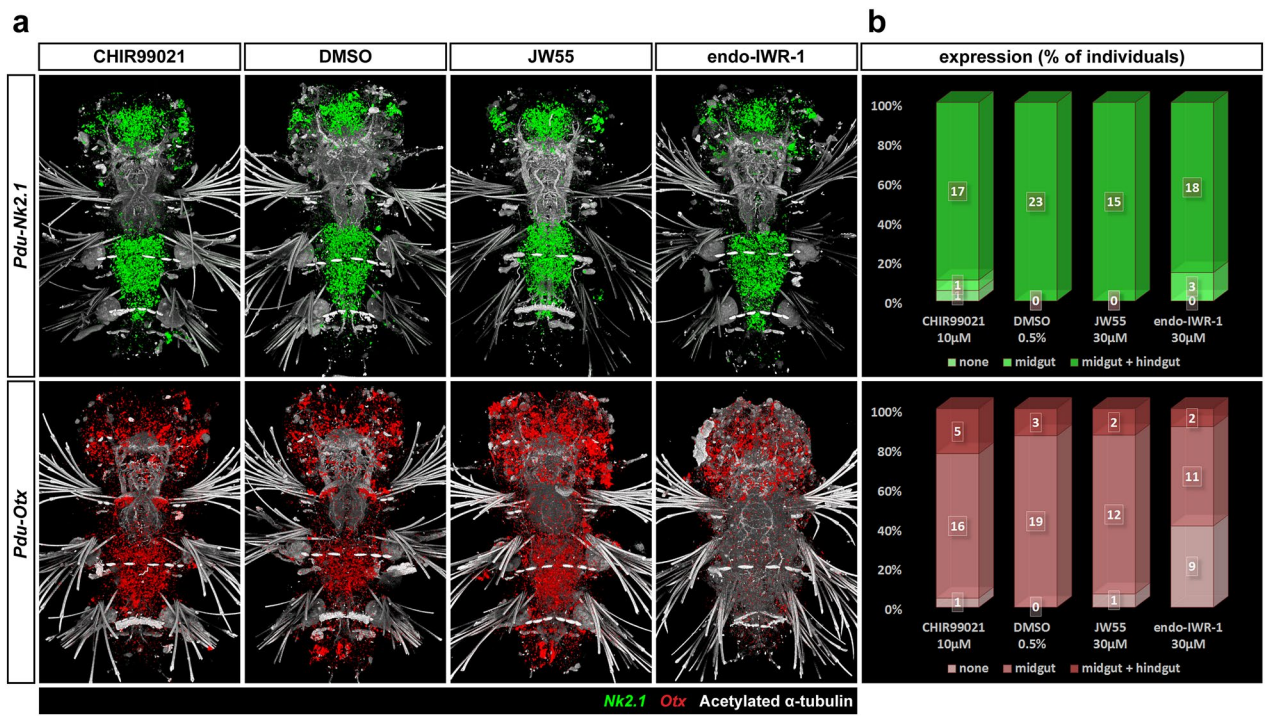

Fig. 10 Expression of endodermal marker genes. **a** Fluorescent in situ hybridization of two endodermal marker genes, *Pdu-Nk2.1* and *Pdu-Otx* and **b** quantification of the effect of activation/inhibition of Wnt/β-catenin pathway on their expression by assignment of all individuals to phenotypic classes. The expression of these genes is typical for neuroectoderm in early larvae but similar to *Pdu-Tcf*, they obtain new expression domains in the gut later in development while still retaining their neurospecific expression. *Pdu-Nk2.1* (top row, green) is expressed in both midgut and hindgut under normal circumstances, and the same pattern is observed in the presence of any of the tested inhibitors or the activator. This is consistent with the theory of midgut to hindgut conversion upon Wnt/β-catenin signalling inhibition since the gene is normally expressed in both tissues. *Pdu-Otx* is typical for mandibular cells, the entire midgut, whereas it is absent from most of the hindgut except for the very posterior cells which form the sides of the anus. Its midgut expression disappears in the presence of the stronger inhibitor (IWR-1-endo), while a normal pattern is observed with the milder JW55 inhibition (which might just point to unsuccessful inhibition on this batch). Neural and mandibular expression is preserved under all conditions, although it is somewhat lower after Wnt/β-catenin inhibition given the overall smaller size of mandibles. Interestingly, the cells surrounding the anus lose *Pdu-Otx* expression also in the presence of either CHIR99021 activator of Wnt/β-catenin signalling

inhibited (Fig. 11). The changes in expression of all studied genes are summarized in Fig. 12.

Discussion

Pdu-Tcf isoforms

It was previously pointed out that a great difference exists between the diversity and specialized functions of vertebrate Tcf proteins with their many isoforms in contrast to the poor *Tcf* gene repertoire in invertebrates (both deuterostomes and protostomes) [16, 21]. Their genomes, with some exceptions (Planaria, Platyhelminthes), usually comprise only one *Tcf* gene, and their repertoire of isoforms has been underappreciated until recently, although their presence in other organisms as *Drosophila* has also been mentioned. Thus, it is not clear whether complex regulation by Tcf proteins is limited only to organisms that possess multiple *Tcf/LEF* genes or whether it is also present in Protostomia with only single *Tcf* gene, and if yes, how it is achieved. Here, we have shown that a large diversity of Tcf isoforms is present in a spiralian which can potentially compensate for the lack of gene diversity in invertebrates. According to our BLAST search, *Tcf* isoforms have been already found in the sequencing of many invertebrate genomes. From vertebrates, the top hits usually belonged to various isoforms of the *Tcf7l2* (*Tcf4*) gene. Since the duplications of *Tcf* genes occurred only after the divergence of *Platynereis* and vertebrate lineages, this does not reflect closer homology than with other vertebrate *Tcf* genes, but it could rather point out to closest functional similarity. It is interesting that *Tcf4* is able to both activate [30] and repress [29] transcription depending on context [28] and it is necessary for renewal of gut epithelium [30], while *Tcf1* and *Tcf3* are considered to be purely an activator or an inhibitor of gene expression, respectively [27]. We hypothesize that Pdu-Tcf is able to undertake both the role of activator and repressor

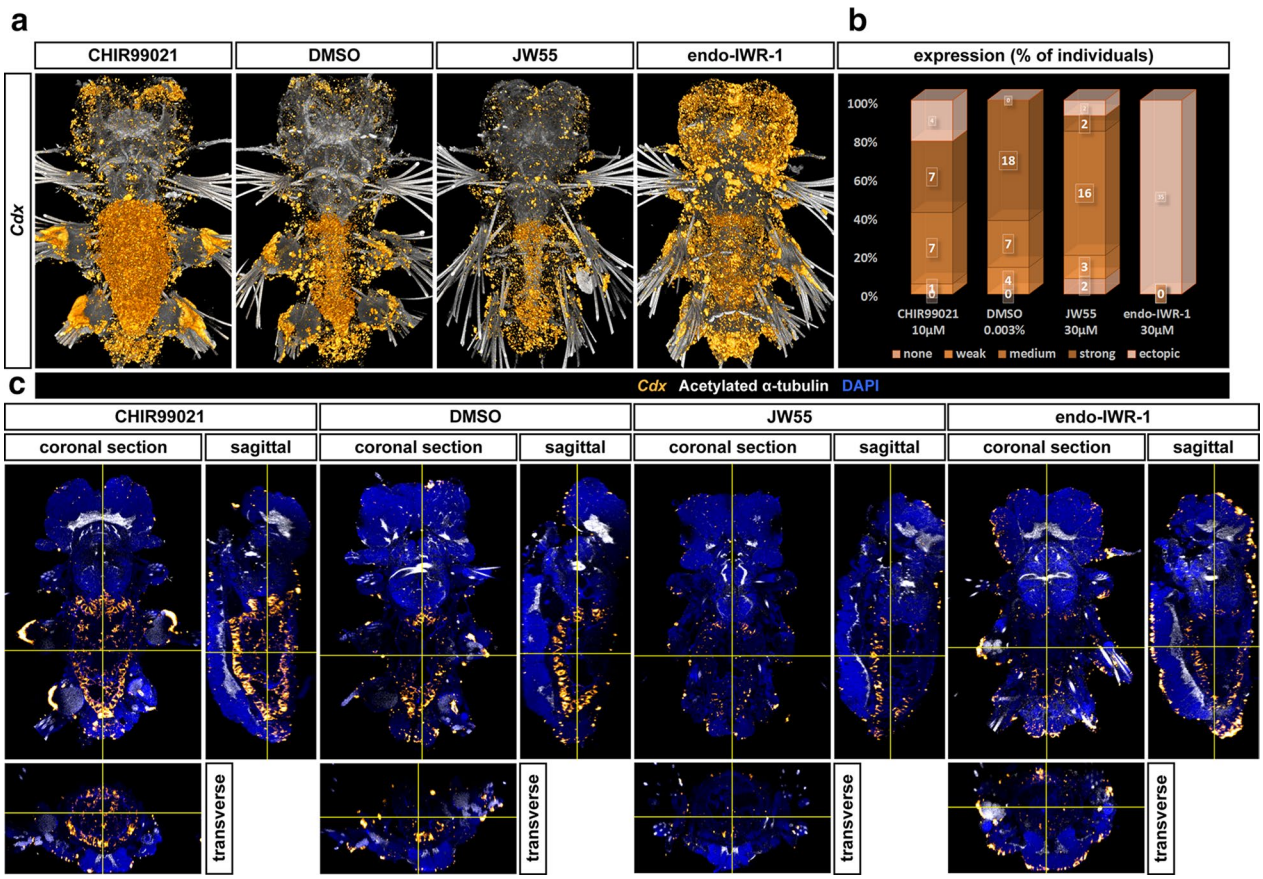

Fig. 11 Expression of *Pdu-Cdx* ParaHox gene. **a** 3D reconstructions of confocal fluorescent in situ hybridization's *z*-stacks detecting a ParaHox gene *Pdu-Cdx* and **b** A graph showing the percentage of individuals assigned to classes according to the level of expression (the darker the higher) upon Wnt chemical treatment. Under control conditions, *Pdu-Cdx* is expressed in the whole hindgut, (in lower level) on the ventral floor of midgut and on the foregut/midgut border. With the Wnt activator (CHIR99021), it was expressed in similar levels but in entire gut, whereas with the inhibitor JW55, the expression is somewhat lower but the pattern remains the same. Widespread ectopic expression with the stronger inhibitor (IWR-1-endo) prevented the proper quantification of phenotypes when using bright-field microscopy. **c** Orthogonal views of the same *z*-stacks reveal the expression in the hindgut and at the midgut/foregut boundary connected by a narrow strip of *Pdu-Cdx* expression on the ventral floor of the gut. This expression expands to most of the gut upon Wnt activation, and the overall expression is lower in the presence of Wnt inhibitors

function. In addition, we propose that individual Tcf isoforms may have slightly different DNA binding characteristics and hence select overlapping, but non-identical sets of target genes and activates or represses them with a different strength relative to each other than another isoform.

Wnt/β-catenin signalling in gut development

The conservation of Wnt/β-catenin signalling's role in digestive tract development still remains questionable due to a lack of detailed data from mostly invertebrate phyla, namely the group Spiralia, which do not contain any of the "classical" model organisms. Cell lineage tracing and fate mapping have been done several times in *Platynereis* in an increasingly greater depth, most recently up to the midtrochophore stage. Expression profiling based on single-cell RNA seq, back-mapping to the larval body based on a reference gene expression atlas and subsequent clustering according to the typical expression fingerprints of major organ systems [84] revealed cell populations in early larva which later grow and differentiate to the gut. It presumably originated from two small clusters of peptidergic cells that express *Hnf4* and symmetrically positioned along the ventral midline on the level between second and third segment. Since *Pdu-Tcf* is expressed broadly in putative endodermal and mesodermal cells in the body of the 48 hpf larva, it is probable that it is expressed in *Hnf4*-positive cells as well. Moreover, *Pdu-Tcf* is present in internal clusters of cells at 72 hpf reminiscent of *Hnf4*+ putative gut

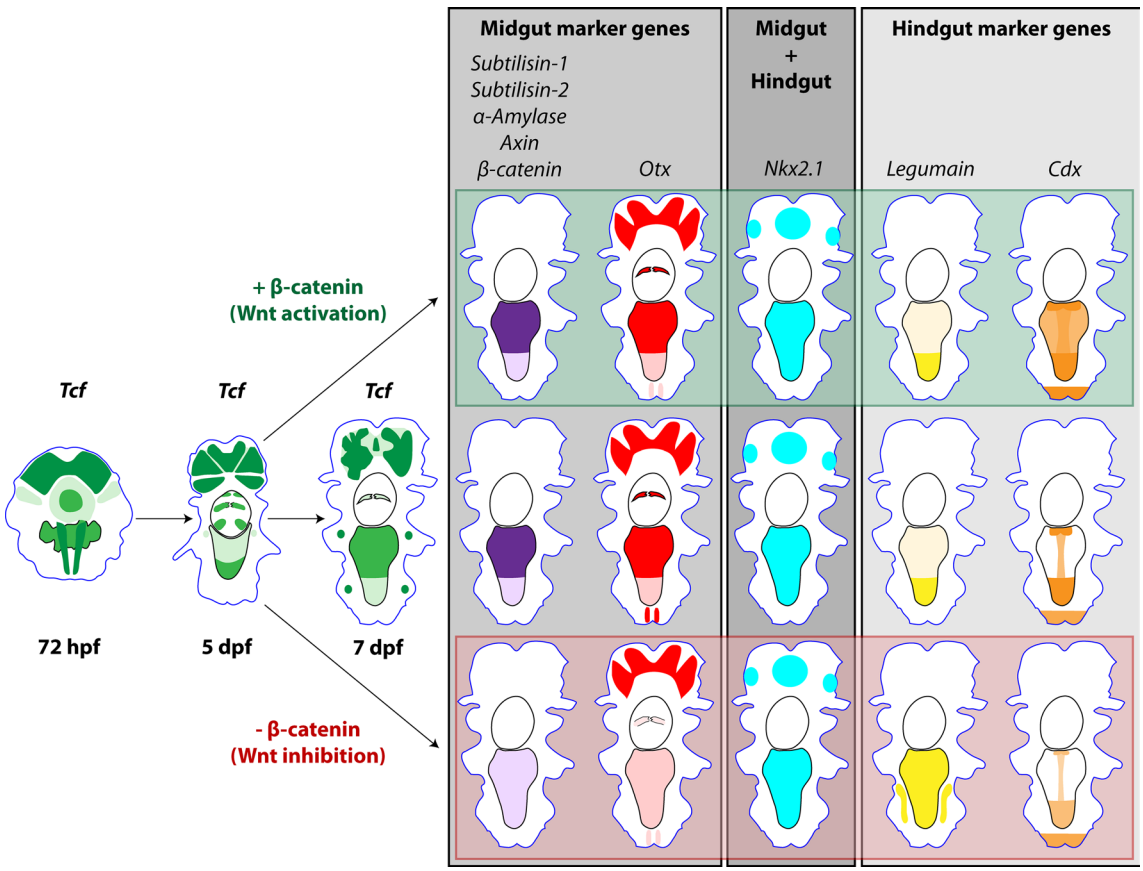

Fig. 12 Schematic representation of *Pdu-Tcf* expression and the role of Wnt/β-catenin signalling in gut development. This diagram summarizes the expression of *Pdu-Tcf* from the 72 hpf stage with putative gut primordium, through 5 dpf stage with uncellularized gut to 7 dpf larva with a functional, compartmentalized digestive tract. Midgut marker genes are generally downregulated by inhibition of Wnt/β-catenin signalling, whereas hindgut marker gene *Pdu-Legumain* expands to midgut. The expression profile of these genes in midgut is thus more reminiscent of the hindgut upon Wnt inhibition. On the other hand, another hindgut gene *Cdx* is slightly downregulated upon inhibition. We did not observe any pronounce effect of Wnt/β-catenin pathway activation, except for *Cdx* which is upregulated in the midgut. The expression of the endodermal marker, *Nk2.1*, which is present in both compartments under normal circumstances, is not changed by either manipulations of the Wnt/β-catenin pathway. According to our scenario, Wnt/β-catenin signalling is necessary for endoderm to differentiate into midgut secretory epithelium and its inhibition causes midgut cells to be functionally converted to the hindgut

precursor cells and might thus represent the developing gut. Hence, it is possible that *Pdu-Tcf* is expressed in the gut from the earliest stages of its development.

Later in development, *Pdu-Tcf* transcripts can be detected in the hindgut and two paired domains (one of them comprising the jaws) in the foregut, but only slightly in the midgut of a 5 dpf larva. This may possibly be due to the fact that the midgut at this stage consists primarily of large not yet cellularized macromeres. Interestingly, this pattern corresponds to the expression of *Tcf4* and *Lef1* in the developing chicken gut, where they are necessary for formation of gizzard microvilli and differentiation of hindgut cells, respectively. However, in the cellularized gut of the 7 dpf larva, *Tcf* is already strongly transcribed in the midgut but less in hindgut evoking more the expression of *Tcf4* in both developing and adult mammalian gut [26, 85]. Not surprisingly, given that *Pdu-Tcf* is expressed in the developing jaws, it is of note that the chitinous mandibles of treated larvae are smaller and less developed. Together with our finding that cell proliferation in the gut (and throughout the whole body) is severely reduced or abolished in larvae treated with a strong Wnt inhibitor, these results suggest that Wnt/β-catenin signalling is necessary for midgut cellularization which requires cell division, reminiscent of the *Tcf4* role in the maintenance of mammalian adult gut epithelium [30].

Expression of endodermal marker gene *Pdu-Nk2.1* shows that the midgut cells of larvae, whose Wnt/β-catenin signalling was inhibited, retained endodermal

characteristics. Yet, the midgut marker genes *Pdu-Subtilisin-1*, *Pdu-Subtilisin-2*, *Pdu-α-Amylase* and *Pdu-Otx*, which are under normal circumstances expressed at high levels in the midgut but absent from the hindgut at 7 dpf, are lost upon pharmacological inhibition of Wnt/β-catenin pathway. At the same time, the hindgut marker gene *legumain* protease precursor expands from hindgut to midgut. Taken together, these two pieces of evidence suggest that midgut cells lose their ability to express digestive enzymes and obtain (or retain) hindgut-like characteristics, when Wnt/β-catenin is inhibited. All changes in gene expression are summarized in the scheme illustrated in Fig. 12.

There is small, but a notable difference between the expression patterns of digestive enzymes reported in our paper and in previous work conducted by Jékely group [41] which reported that *Pdu-Subtilisin-1* and *Pdu-Legumain* are expressed in both the midgut and the hindgut, whereas we found that *Pdu-Subtilisin-1* was predominantly a midgut gene and *Pdu-Legumain* was expressed strongly in the hindgut but only very weakly in the midgut (although some inter-individual variance among larvae is already present at this life stage and some individuals with expression in both gut compartments can be found). The likely explanation for this discrepancy comes from the fact that Williams and colleagues described the expression on 6 dpf stage, when cellularization of the gut is still occurring and the differentiation of the gut has not been completed. Initially, the midgut and hindgut cells are more alike, and only at 7 dpf stage, the expression patterns become more resolved with most of the digestive enzymes expressed in the broad midgut, while the narrow hindgut is dedicated more to defecation than digestion.

Pdu-Cdx can also be considered a hindgut gene, but unlike *Pdu-Legumain,* we saw quite the opposite effect on its expression, which was downregulated upon Wnt inhibition and upregulated in the midgut in the presence of a Wnt activator. *Pdu-Cdx* is a direct target that is activated by Wnt/β-catenin signalling [8]. On the other hand, the digestive enzymes are probably indirect targets which can be upregulated by the decrease in their transcriptional repressors, themselves under the control of Wnt/β-catenin signalling, and can thus react to Wnt inhibition opposite than *Cdx*. The transcription of *Cdx* genes is fully triggered only by Tcf proteins that have a functional, intact C-clamp [36]. Although we do not know which *Pdu-Tcf* isoform is expressed in the gut, the involvement of a C-clamp (−) isoform might explain why *Pdu-Cdx* transcription is not activated in the midgut under normal circumstances, with the exception of the ventral floor and both foregut and hindgut borders that are closest to Wnt sources.

The observation that chemical manipulation of the Wnt/β-catenin pathway from 5 to 7 dpf does affect the expression of the Wnt target gene *Pdu-Axin*, but not dramatically, although reported to be the case at 55 dpf [46], which may be explained by the fact that vertebrates have two *Axin* genes and only *Axin2,* but not *Axin,* has been shown to be regulated by Wnt/β-catenin signalling [17] even though they are functionally equivalent in vivo [86]. On the other hand, *Platynereis* has most probably only one *axin* gene, whose responsiveness to Wnt/β-catenin signalling might be time and tissue dependent. Immunostaining against β-catenin clearly shows that the stronger inhibitor IWR-1-endo indeed reliably downregulates Wnt/β-catenin signalling and β-catenin staining and the effects on gene expression shared with IWR-1-endo suggest that the other inhibitor, JW55, works as well but produce milder phenotypes at the same concentration. The reliability of the activator CHIR99021 remains questionable since it always produced mild or no effects at this stage (except the missing *Pdu-Otx*-positive cells of the anus) although it works very well on younger larvae (our observation, data not shown). The absence of strong phenotypic and gene expression differences in the presence of the activator can also suggest that the Wnt/β-catenin signalling has a permissive instead of an instructive role in gut development. In such a scenario, the pathway's activity is required, but not sufficient for the gut endoderm to acquire midgut fate and gene expression fingerprint. Therefore, other transcriptions factors and signalling pathways would have to be involved to pattern the developing gut as has been shown for Hox genes [87], Hedgehog [88, 89], BMP [90] and Notch signalling [91] in other organisms.

Based on preliminary results, it seems that *Pdu-Tcf* itself might be a target of Wnt/β-catenin pathway. Upon Wnt activation, it is strongly transcribed in both the midgut and the hindgut, whereas the expression is lower after inhibition. This points to a positive feedback loop to reinforce *Pdu-Tcf* expression in the gut, where high amounts might be necessary. Positive autoregulation has been described before for LEF1 in colon cancer [92], XTcf-4 (Tcf7l2) in *Xenopus* midbrain [93] and zebrafish Tcf3 (Tcf7l1) [95]. It is achieved through the direct binding of Tcf to its promoter [94, 96], but, in vertebrates, this can be mediated by a different Tcf [97]. Interestingly, there was higher and broader *Pdu-Tcf* expression in the presence of Wnt/β-catenin inhibitors in the rest of the body, e.g. brain ganglia. This could be explained by the presence of a negative feedback loop to maintain steady and localized *Pdu-Tcf* expression. Such dual regulation in two different tissues, if confirmed, could be achieved by the action of tissue-specific sets of transcriptional cofactors. Further investigation will be needed to verify these

results and ascertain the real situation about *Pdu-Tcf* autoregulation.

The differences between the midgut and the hindgut and the observed midgut to hindgut transition caused by inhibition of Wnt signalling (Fig. 12) probably cannot be accounted for *Pdu-Tcf* alone. Our in situ hybridization stainings show that it is expressed in the hindgut and some parts of foregut at 5 dpf, whereas it is present in both midgut and less in hindgut later at 7 dpf. The same is true for *Pdu-Axin* at 7 dpf. If we take a look at immunofluorescence staining of β-catenin as a proxy of Wnt/β-catenin pathway activity, we see it is present in high amounts in the midgut but not in the hindgut. This difference might be the results of the combinatorial action of factors involved in Wnt/β-catenin signalling cascade or in the modulation of Wnt signal, e.g. sources of Wnt proteins, expression of Frizzled receptors or soluble Wnt inhibitory proteins. When the Wnt/β-catenin pathway is inhibited, β-catenin diminishes from the midgut, whereas no change occurs in the hindgut, since β-catenin levels there are naturally low. As a result, the gene expression regulated by Wnt signalling via β-catenin in both parts of the gut exhibits hindgut-like characteristics.

Conclusions

In this paper, we described a single *Tcf* homologue in the marine polychaete annelid *Platynereis dumerilii*. It produces an array of mRNA variants via alternative splicing with a potentially different DNA binding capacity and function. *Pdu-Tcf* and several other components of Wnt/β-catenin signalling pathway (*Axin*, β-catenin) are present in the developing gut of *Platynereis* and inhibition of Wnt/β-catenin signalling causes the midgut to obtain a hindgut-like expression of digestive enzymes and leads to a loss in cell proliferation. Taken together, *Platynereis* appears to be a useful model to investigate the roles of Wnt/β-catenin signalling in organ development in a relatively simple system and to find features of gut differentiation and maintenance that have been conserved in Bilateria.

Authors' contributions

ZK proposed the project. RZ, ZK and MO designed the experiments. RZ performed the experiments. RZ analysed the sequences and RZ with ZK wrote the manuscript. All authors read and approved the final manuscript.

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Acknowledgements

We thank Jasper E. Manning, Jiri Pergner and two anonymous referees for critically reading the manuscript and for their helpful comments. We thank Chrysoula Pantzartzi for help with identification of C-clamp variant of *Pdu-Tcf* and for consultations on *Pdu-Tcf* sequence, Veronika Nosková for taking care of our *Platynereis* cultures, Gáspár Jékely, Detlev Arendt and David K. Ferrier who kindly provided us with templates of in situ probes and all members of the Kozmik's laboratory for their support and suggestions.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The original data and materials supporting conclusions in this article, i.e. z-stacks from images, the complete sets of bright-field photographs of all larvae assigned to groups according to their marker gene expression and the templates used for generation of in situ hybridization probes, are available from the corresponding author on reasonable request. The novel sequences used to support the conclusions of this article are available in the NCBI's GenBank Nucleotide repository https://www.ncbi.nlm.nih.gov/genbank/ [99] under following accession numbers: *Pdu-Tcf* isoform X7, complete sequence (MG952772), *Pdu-Tcf* N-terminal probe (MG952774), *Pdu-Tcf* isoform X1 C terminus (MG952775), *Pdu-Tcf* isoform X2 C terminus (MG952776), *Pdu-Tcf* isoform X3 C terminus (MG952777), *Pdu-Tcf* isoform X4 C terminus (MG952778), *Pdu-Tcf* isoform X5 C terminus (MG952779), *Pdu-Tcf* isoform X7 C terminus (MG952780), *Pdu-Tcf* genomic sequence, C terminus (MG952773).

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

This work was funded by the Czech Science Foundation (17-15374S) and Grant Agency of the Charles University in Prague (98213).

Publisher's Note

Received: 27 February 2018 Accepted: 20 April 2018
Published online: 11 June 2018

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